

Novel treatment strategies for the protection of the preterm brain

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Novel treatment strategies for the protection of the preterm brain

Re-balancing inflammation and regeneration



Daan Ophelders

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Novel treatment strategies for the protection of the preterm brain

Re-balancing inflammation and regeneration

PROEFSCHRIFT

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Abbreviations

aEEG	amplitude-integrated electroencephalogram
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA	analysis of variance
APC	antigen presenting cells
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BPD	bronchopulmonary dysplasia
BRS	baroreceptor reflex sensitivity
BSA	bovine serum albumin
BW	body weight
<i>C. albicans</i>	<i>Candida albicans</i>
CD	cluster of differentiation
cEEG	conventional electroencephalogram
CFU	colony forming units
CI	confidence interval
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS	central nervous system
CP	cerebral palsy
CSF	cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DAMPs	damage-associated molecular patterns
DTT	dithiothreitol
ECG	electrocardiogram
EEG	electroencephalogram
ELISA	enzyme-linked immunosorbent assay
EVs	extracellular vesicles
F	Fluconazole
FDR	false discovery rate
FHR	fetal heart rate
FIRS	fetal immune response syndrome
GA	gestational age
GFAP	glial fibrillary acidic protein
GVHD	graft-versus-host-disease
H&E	hematoxylin & eosin
H ₂ O ₂	hydrogen peroxide
HCl	hydrochloric acid
HI	hypoxia-ischemia

HIE	hypoxic-ischemic encephalopathy
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
IA	intra-amniotic
IBA-1	ionized calcium-binding adaptor molecule-1
IFN γ	interferon gamma
IL	interleukin
IP	intra-peritoneal
IQR	interquartile range
IR	immunoreactivity
MABP	mean arterial blood pressure
MAPC	multipotent adult progenitor cell
MBP	myelin basic protein
MHC	major histocompatibility complex
MPAQ	Maastricht-programmable acquisition system
MPO	myeloperoxidase
MSC	mesenchymal stromal cells
MSC-EVs	mesenchymal stromal cell-derived extracellular vesicles
NaCl	sodium chloride
NEC	necrotizing enterocolitis
NICU	neonatal intensive care unit
NiDAB	nickel sulfate-diaminobenzidine
NMDA	N-methyl-D-Aspartate
NTA	Nanoparticle Tracking Analyses
Olig2	oligodendrocyte transcription factor 2
ovRPS15	ovine 40S ribosomal protein S15
PAMPs	pathogen-associated molecular patterns
PAS	periodic acid-schiff
PBS	phosphate buffered saline
PBST	tween in phosphate buffered saline
PEG	polyethylene glycol
PPROM	preterm premature rupture of the membranes
preOLs	oligodendrocyte precursors
PRR	pattern recognition receptors
PVDF	polyvinylidene difluoride
PVL	periventricular leukomalacia
PVWM	periventricular white matter
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROP	retinopathy of prematurity

ROS	reactive oxygen species
RT-qPCR	real time quantitative polymerase chain reaction
SAL	saline
SBP	systolic blood pressure
SCWM	subcortical white matter
SD	standard deviation
SD-RR	standard deviation of the mean R-R interval
SDS	sodium dodecyl sulfate
SD-SBP	standard deviation of the mean systolic blood pressure
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
T cell	T-lymphocyte
Treg	regulatory T-lymphocyte
TBS	tris-buffered saline
TMB	3,3',5,5'-tetramethylbenzidine
TNF α	tumor necrosis factor alpha
Tsg101	tumor susceptibility gene 101
UCO	umbilical cord occlusion
WM	white matter

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1

General Introduction

PRETERM BIRTH

Preterm birth is defined as birth before 37 weeks of gestation and is the leading cause of perinatal mortality and morbidity in developed countries.¹ In the Netherlands, approximately 7.5% of all infants are born preterm.² In contrast to increasing numbers of preterm births world-wide, in the Netherlands, preterm birth numbers have stagnated over the last years.²

Survival rates following preterm birth have sharply increased over the last decades. This increase can be attributed to improved early perinatal and neonatal care, such as improved prenatal diagnostics, the use of antenatal corticosteroids, postnatal administration of exogenous surfactant, centralization of perinatal care, and advanced ventilator strategies.^{3,4} Upon birth, almost all preterm neonates require medical care to support function and further development of their immature organs. The kind and intensity of medical care for preterm babies is mostly determined by their gestational age and corresponding degree of developmental immaturity of their organs. This immaturity results in the incapacity to deal with the challenges of life outside the uterus, mostly oxygen and inflammation, and the underlying cause of preterm birth. Despite improved medical care, preterm birth remains associated with development of diseases of prematurity such as necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), and periventricular leukomalacia (PVL) resulting in cerebral palsy (CP).⁵ From these diseases, injury to the preterm brain (PVL and CP) is the biggest contributor to long-term morbidity in preterm infants.⁶ During gestation, complex differentiation and maturation processes contribute to fetal brain development in a spatial- and time-dependent fashion. For instance, white matter myelination follows a caudo-rostral gradient and progresses from the center to the periphery.^{7,8} Preterm infants are born during this critical developmental period, in which the fetal brain is particularly vulnerable to exogenous and endogenous stressful events, such as intra-amniotic infection/inflammation and hypoxia-ischemia. These events might affect normal brain ontogenesis and may cause irreversible injury to the brain.

CAUSES OF PRETERM BIRTH

Preterm birth can be subdivided in distinct categories: (1) medically indicated preterm birth in which maternal and/or fetal conditions prompt an immediate intervention at preterm gestational ages (preeclampsia, intrauterine growth restriction, and placental abruption conditions) and (2) spontaneous preterm birth with intact membranes; or (3) preterm premature rupture of the membranes (PPROM).¹ Many different causes can trigger spontaneous premature uterine contractions leading to preterm birth, including

(1) activation of the maternal/fetal hypothalamic-pituitary-adrenal (HPA) axis as a result of maternal and/or fetal stress, (2) infection and inflammation, (3) hypoxia-ischemia and (4) uterine over distension (for instance during a multiple pregnancy).^{1, 9-11} From these causes infection and inflammation and hypoxia-ischemia are most frequently diagnosed and will be subject of this thesis.^{1, 9-11}

The uterine environment has long been considered to be a sterile environment. With the availability of advanced microbiological methodologies, we are now aware that numerous microbial organisms colonize the uterine environment, some of which have been causally linked to preterm birth.¹² Importantly, bacterial colonization of the amniotic cavity does not necessarily induce preterm birth.¹³⁻¹⁶ Timing of bacterial colonization of the amniotic cavity rather than colonization itself is a key factor for the risk of preterm birth.^{14, 16} Therefore, the percentage of diagnosed intra-uterine infections is much higher in very preterm infants compared to near term and term infants.^{10, 17-19}

Nonetheless, bacterial colonization without induction of preterm birth remains sufficient to cause adverse fetal development and cause injury to fetal organ systems.¹⁶ Such an intra-amniotic infection induces an inflammatory response of the chorionic and amniotic membranes, which is referred to as chorioamnionitis. Various routes have been described by which microorganisms gain access to the amniotic cavity: (1) ascending from the vagina and the cervix, which is the most common pathway; (2) hematogenous dissemination through the placenta; (3) accidental introduction at the time of invasive procedures (i.e. amniocentesis); (4) retrograde spread through the fallopian tubes; and (5) asymptomatic endometrial colonization before pregnancy.²⁰

A wide variety of microorganisms, comprising bacteria, viruses, and fungi/yeasts have been identified in the amniotic fluid and membranes of preterm infants.²¹⁻²³ It is unclear during which part of conception, implantation and gestation intra-uterine infection or microbial invasion occurs. Most women do not show clinical symptoms of chorioamnionitis which is referred to as silent chorioamnionitis and is only diagnosed at birth by histological examination. A small proportion of pregnant women with preterm birth, however, show clinical signs of chorioamnionitis such as maternal fever, uterine fundal tenderness, maternal tachycardia, fetal tachycardia, and purulent or foul amniotic fluid. Upon diagnosis of this clinical chorioamnionitis preterm birth is often medically indicated, whereas asymptomatic chorioamnionitis often results in spontaneous preterm birth.

The mechanisms by which intrauterine infection / inflammation lead to preterm birth comprise activation of the innate immune system: recognition of pathogen-associated molecular patterns (PAMPs), expressed by invading microorganisms, by pattern recognition receptors (i.e. toll-like receptors) elicits the release of pro-inflammatory mediators such as interleukin (IL)-8, IL-1 β , and tumor necrosis factor (TNF)- α .¹ Microbial

endotoxins and pro-inflammatory cytokines stimulate the production of prostaglandins, other pro-inflammatory mediators, and matrix-degrading enzymes. Prostaglandins stimulate uterine contractility, whereas degradation of extracellular matrix in the fetal membranes leads to PPROM.^{1, 10}

When the fetus is directly exposed to intra-amniotic inflammation / infection (i.e. skin contact or swallowing and breathing movements *in utero* or via the placental-fetal circulation), recognition of PAMPs by the fetal innate immune system might result in an elevation of levels of pro-inflammatory cytokines.^{24, 25} This response is referred to as Fetal Immune Response Syndrome (FIRS).²⁴ Intrauterine infection/inflammation is causally involved in the induction of organ injury, including the brain. Although the central nervous system is an immune-privileged site, separated from the systemic circulation by the blood brain barrier, microglia, the resident immune cells of the central nervous system, can sense inflammatory signals in the systemic circulation through: (1) interaction of systemic molecules with cerebral immune cells in the circumventricular organs: areas that are devoid of a blood brain barrier²⁶⁻²⁹; (2) Direct access of inflammatory mediators or cells into the central nervous system facilitated by breakdown of the blood brain barrier²⁶⁻²⁹; (3) interactions of systemic molecules with endothelial cells resulting in release of inflammatory mediators (i.e. prostaglandins) at the basal side into the brain parenchyma.²⁶⁻²⁹

HYPOXIA-ISCHEMIA

Fetal hypoxia-ischemia (HI) is the result of impaired gas exchange between mother and fetus. Causes for fetal HI are inadequate placental perfusion due to maternal complications, impaired umbilical cord blood flow due to mechanical compression, rupture or prolapse of the umbilical cord. In the case of hypoxia-ischemia preterm birth is mostly medically indicated.

Regardless of the cause of the hypoxic insult, cardiac and vascular compromise will ultimately occur when hypoxia is prolonged. This results in hypotension, ischemia and anaerobic metabolism. Ischemia therefore is both the cause and a consequence of hypoxia leading to diminished delivery of nutrients and impaired removal of metabolites. The process of hypoxia-ischemia resulting in energy failure and subsequent brain injury is a process that evolves over time and can be subdivided into two main phases^{30, 31}: (1) primary phase of injury and (2) secondary phase of injury.

During the initial phase cerebral oxidative metabolism is severely compromised resulting in depletion of adenosine triphosphate (ATP) and uncontrolled membrane polarization allowing for cellular influx of calcium and subsequent proteolytic degradation of multiple cellular structures. Neuronal release of glutamate accompanied

by impaired removal of extracellular glutamate by astrocytes causes its accumulation and activation of *N*-methyl-D-Aspartate (NMDA) channels, α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate receptors, resulting in acute cell swelling, production of reactive oxygen (ROS) and reactive nitrogen species (RNS), and NMDA receptor-mediated seizure activity.³⁰⁻³²

After reperfusion, the initial induced accumulation of excitatory amino acids resolves with apparent recovery cerebral oxidative metabolism. Although short in time, the neurotoxic cascade is largely inhibited during this period, thereby providing a “therapeutic window of opportunity” for neuroprotective therapies.³³

Although restoration of blood and oxygen supply to the brain is necessary to limit the hypoxic-ischemic brain damage, reperfusion exacerbates cell injury in the secondary phase. Intracellular increase of calcium during hypoxia-ischemia and increased oxygen levels during reperfusion are involved in formation and accumulation of ROS and RNS. Production of free radicals results in oxidation of cellular components such as membrane lipids, proteins, and DNA, leading to mitochondrial injury cellular damage, and cell death by either apoptosis or necrosis. Whereas apoptosis is an orchestrated process of cell death that causes negligible effects on the extracellular environment, necrosis caused disruption of cell-membrane integrity and leakage of cellular components that function as danger-associated molecular patterns (DAMPs) to trigger a sterile (non-infectious) inflammatory response.³⁴

Despite differences in etiology (infectious versus sterile inflammation), microglia are at the convergence point in the neuroinflammatory cascade leading to preterm brain injury.^{29, 35} Microglia are the resident macrophages of the central nervous system, and have the ability to recognize a wide range of signals (i.e. PAMPs and DAMPs) that indicate a threat to the structural and functional integrity of the CNS through various pattern recognition receptors (PRRs), glutamate receptors, and purinergic receptors, resulting in activation.³⁶⁻³⁸ Microglial activation is necessary for destruction of microorganisms, clearance of cellular debris and repair of tissue. However, uncontrolled activation of microglia can cause damage to neighboring neurons and oligodendrocytes. Upon activation, microglia change their morphology from a ramified to a hyperramified and subsequent amoeboid morphology.³⁸ These morphological changes are accompanied by a robust production of pro-inflammatory cytokines, reactive oxygen and nitrogen species, excitotoxic molecules, and proteolytic enzymes.^{38, 39} Moreover, microglia act as antigen presenting cells (APCs) through upregulation of HLA molecules, co-stimulation and cytokine production, thus providing signals necessary for activation of T-lymphocytes (T cells).⁴⁰

Astrocytes also play a crucial role in the brain. These cells are key in regulating secretion/absorption of neurotransmitters, transmission of electrical impulses, maintenance of the blood-brain barrier, providing nutrients to neuronal tissue and are

involved in glial scar formation after damage. Moreover, reactive astrocytes are an important source of pro-inflammatory cytokines that affect neurons and oligodendrocytes.^{41, 42}

Under inflammatory conditions, the blood-brain barrier is disrupted and CNS immune privilege is greatly undermined, allowing for active recruitment of circulating leukocytes, including monocyte-derived macrophages, neutrophils and T cells. These systemic immune cells contribute to the neuroinflammatory response and subsequent injury of the preterm brain.⁴³⁻⁴⁵

Preterm brain injury is mainly characterized to injury of the cerebral white matter. Myelination of axons by mature oligodendrocytes is essential for optimal signal transduction and subsequent brain function and development. During gestation, cerebral white matter is predominantly populated by oligodendrocyte precursors (preOLs) that develop towards mature, myelin producing, oligodendrocytes. At this stage preOLs are most vulnerable to excitotoxic, oxidative, and inflammatory injury, resulting in maturational arrest or cell death and subsequent hypomyelination, characteristic for preterm brain injury.^{6, 46-48}

During maturation preOLs have low levels of anti-oxidants, making them particularly sensitive for excessive production of free radicals. In addition, during the peak period of vulnerability maturing preOLs overexpress the AMPA receptor that is lacking the GluR2 subunit. Deficiency of this subunit makes the preOL calcium-permeable upon stimulation with glutamate, thereby causing intracellular generation of ROS/RNS.³⁵

The main form of white matter injury is periventricular leukomalacia (PVL), which can be sub-divided into two forms.⁴⁸ Focal (cystic) PVL is characterized by macroscopic necrotic lesions and loss of all cellular elements. These necrotic lesions evolve into cystic lesions that can be visualized by cranial ultrasound. However, the incidence of focal PVL has decreased tremendously and nowadays non-cystic PVL is the most common variant of PVL.^{6, 48} Non-cystic PVL is characterized by diffuse spread of microscopic necrotic foci with concomitant astro- and microgliosis and a decrease in premyelinating oligodendrocytes. This decrease is often counteracted by an increase in oligodendrocyte progenitor cells. However, these cells often lack processes and do not seem to have the capacity to fully mature into myelin-producing oligodendrocytes.^{6, 46, 48}

GRAY MATTER

Although white matter injury is the hallmark of preterm brain injury, accumulating evidence suggest an increasing role for gray matter in the outcomes of preterm infants suffering from preterm brain injury.⁶ Depending on the gestational age and injurious insult, reduction in growth of and significant neuronal loss in cortical and subcortical

gray matter structures, including the basal ganglia, thalamus, hippocampus, and cerebellum are identified.⁴⁹ In addition, subplate neurons, a transient cell population, required to establish thalamo-cortical projections, are vulnerable to prenatal insults. Disturbance of formation of thalamo-cortical projections is a critical risk factor for adverse neurocognitive outcomes.^{6, 50, 51}

DIAGNOSIS

Imaging techniques are frequently used for the detection of white matter abnormalities during the early neonatal period. Although minimally invasive, weeks or more are required for structural injury to become evident on ultrasonography. As preterm brain injury often presents with clinical and electrophysiological seizures, assessment of brain function with electroencephalography (EEG) provides a powerful, sensitive and non-invasive tool for the assessment of brain injury in these infants.⁵²⁻⁵⁴

EEG records the cerebral electrical activity of the brain through electrodes attached to the scalp. Although, conventional EEG (cEEG) is the gold standard for evaluating the neurologic functional status of preterm neonates in the neonatal intensive care unit (NICU), it presents some difficulty because of the involved technical complexity, time demands, intermittent availability, interpretation specialty, and costs.⁵⁵ Amplitude-integrated EEG (aEEG), in contrast to cEEG, is a simplified method of continuous brain function monitoring that displays trended brain activity and is highly correlated with cEEG in monitoring background activities of both healthy and neurologically compromised neonate.^{52, 56} aEEG consists of a single- or double-channel EEG that is recorded by three to five electrodes attached to the scalp.⁵⁷ It continuously captures the general background pattern of the brain activity in real time for an extended period. Recordings can last for 24 hours or longer if needed. The electrode application and aEEG interpretation entail relatively short training and experience to master. The benefit of longer-term recordings by aEEG may be useful for capture of episodic seizure activity and evolving trends in background activity after acute injury and to monitor therapeutic responses.⁵⁸

Besides assessment of electrocortical cortical and background activity with aEEG, analysis of activity of deep brain structures (i.e. brainstem) might provide an additional diagnostic tool for neonatal outcome as crucial basic functions (i.e. baroreceptor reflex) are located in this structure. The baroreflex is primarily responsible for short-term autonomic control of blood pressure and minimizes fluctuations in by increasing or decreasing heart rate, myocardial contractility, and arterial vascular resistance.⁵⁹

Infants born preterm have a reduced baroreceptor reflex sensitivity (BRS) at birth compared to term-born infants. Moreover, preterm birth alters the normal maturation

of BRS, with reduced BRS in preterm infants at term-equivalent age compared with newborn term infants.⁵⁹

The clinical relevance of an impaired baroreceptor reflex function relates to the increased risk for developing additional brain injury by exposing the vulnerable developing cerebral vascular network to (large) fluctuations in blood pressure. In preterm infants, fluctuations associated with high blood pressure may disrupt the cerebral capillaries in the germinal matrix while periods of low blood pressure may cause localized hypoxia-ischemia of the watershed areas.⁶⁰ Moreover, preterm infants display fluctuating pressure-passivity between systemic blood pressure and cerebral blood flow representing a considerably increased risk of cerebral hemorrhage or hypoxia.⁶⁰

TREATMENT STRATEGIES

Following birth, all preterm infants receive supportive care for adequate respiratory support, optimizing blood gases and blood pressure, normalizing fluid-electrolyte balance and blood glucose levels, and, if necessary, antibiotic treatment and treatment of seizures. Preterm brain injury evolves over time in a period of hours to weeks, creating a window of opportunity. However, therapeutic options to improve the outcomes of preterm neonates suffering from brain injury are very limited.

Current approaches to treat the consequences of chorioamnionitis focus on the symptoms or risk factors rather than the causes. More precisely, treatment of chorioamnionitis aims to induce or augment labor with adequate postnatal support. However, intrapartum therapy with intravenous antibiotics has shown to decrease neonatal sepsis and subsequent adverse outcomes.⁶¹ Moreover, amnion infusion with antibiotics has been used to treat established chorioamnionitis.⁶¹ Postulated mechanisms are dilution of infective organisms and antimicrobial effects of the fluid infused.⁶¹ Although amnion infusion with antibiotic treatment might protect against adverse fetal outcomes, including neurodevelopmental outcomes, this therapy has been aimed at eradication of bacteria, whereas other microorganisms (i.e. fungi and viruses) are not be affected by antibiotic treatment. *Candida albicans* (*C. Albicans*) has frequently been isolated from amniotic fluid of women with preterm labor, and intra-amniotic *C. Albicans* can cause fetal death or fetal candidiasis with impaired neurodevelopmental outcomes.⁶²⁻⁶⁴

Recently, in two clinical cases of intra-amniotic *C. Albicans* infection, treatment with intra-amniotic injections of Fluconazole, supplemented with oral and vaginal treatment resulted in prolonged pregnancy and survival of both neonates.⁶⁵ Moreover, animal

studies have demonstrated that intra-amniotic Fluconazole treatment reduced the fetal systemic inflammatory response elicited by intra-amniotic *C. Albicans*.^{62, 66}

Currently hypothermia is the only established neuroprotective intervention for hypoxia-ischemia-induced injury of the term or late-preterm neonate, with modest protective effects if started within 6 hours after the insult. Preterm infants are excluded from this therapy due to an increased risk of adverse effects, illustrating a great demand for novel therapeutic interventions for preterm brain injury.⁶⁷

BONE MARROW-DERIVED PROGENITOR CELLS

Cell-based interventions as a therapeutic strategy for injury to the (neonatal) central nervous system have gathered much attention during the past decade.⁶⁸⁻⁷⁶ Many different types of stem cells, derived from fetal, placental, and adult tissues are currently under investigation.⁷⁷⁻⁸² Mesenchymal stromal cells (MSCs) are a subset of progenitors that have been shown to differentiate into multiple lineages (i.e. osteoblasts, adipocytes and chondrocytes).⁷⁷⁻⁸⁰ They have been the focus of research as they are easily obtainable (i.e. from cord blood, Wharton's jelly, bone marrow) and do not have the ethical and safety concerns of embryonic stem cells.^{73, 76} Moreover, MSCs have low immunogenicity due to a lack of expression of MHC class II antigens, allowing the use for allogenic therapy.⁷⁶ The therapeutic potential of MSCs is mainly attributed to their immune-modulatory and regenerative potential.^{73, 74, 79, 83} The effects of MSCs on the immune system comprise both modulation of innate and adaptive immune responses from a pro-inflammatory status towards and anti-inflammatory status, thereby reducing tissue injury and creating an environment in favor of tissue repair and regeneration.^{79, 80, 84}

Besides immune modulation in favor of regeneration, MSCs directly affect the injured central nervous system through secretion of neurotrophic factors that stimulate and maintain neurogenesis of the endogenous neuronal stem cell population and subsequent differentiation into neuronal and oligodendroglial lineage.^{74, 79, 83, 85-87} Recently, the neuroprotective potential of systemically administered MSCs was shown in a preclinical ovine model of global hypoxia-ischemia-induced injury of the preterm brain.⁸⁸ MSC administration in the therapeutic window of opportunity established functional improvement and prevented hypomyelination of the subcortical white matter. These protective effects were attributed to anti-inflammatory effects since neuroinflammation and peripheral immune activation were significantly reduced.⁸⁸

However, clinical application of mesenchymal stromal cells requires large cell numbers within the limited time-frame of the window of opportunity. These criteria cannot be

met by autologous transplantation and mandate an off-the-shelf MSC product for allogeneic transplantation.

Recently, multipotent adult progenitor cells (MAPCs), a subpopulation bone marrow derived stromal cells, have been described and characterized. Compared to MSCs, MAPCs have a more pronounced expansion potential, with stable genetics. In addition, MAPCs express lower levels of MHC class I molecules compared to MSCs.⁸⁹⁻⁹¹ Similar to MSCs, MAPCs have immune-modulatory potential.^{90, 91} Moreover, a major obstacle for intravenous administration of cell-based therapies remains pulmonary passage. Fischer et al. demonstrated that due to their smaller size twice as many MAPCs bypass the pulmonary capillary bed compared to larger MSCs after intravenous injection.⁹²

Pre-clinical animal studies clearly have demonstrated therapeutic benefits of MAPCs after traumatic brain injury and spinal cord injury. Moreover, in a murine model of ischemic stroke, intracranial injections of MAPC or MSCs revealed that MAPC had stronger effects on the attenuation of the inflammatory response and more potency to promote endogenous tissue regeneration than MSCs.⁹³

Because of their extensive proliferation potential with stable genetics, low immunogenicity, and therapeutic efficacy, MAPCs are excellent candidates for large scale production and “off the shelf” administration.⁹⁴ Off-the-shelf availability would be of great value with respect to the window of opportunity for treatment in that it makes cell-harvesting for allogeneic transplantation redundant.

The therapeutic potential of both MSCs and MAPCs largely has been attributed to release of paracrine mediators, stored in extracellular vesicles (i.e. exosomes (70-150 nm in diameter) and microvesicles (100-1000 nm)), rather than homing of cells and subsequent substitution of lost cell types.⁹⁵ This paracrine hypothesis is confirmed by the fact that transplanted cells were trapped within the lungs and minor fractions could be detected in the brain following systemic administration.^{88, 96, 97}

Exosomes are small membrane vesicles that arise from multivesicular endosomes and are released into the environment upon fusion with the plasma membrane.⁹⁸ They contain coding and noncoding RNAs, lipids, and proteins. Originally thought to assist in trafficking of extracellular molecules to lysosomes for degradation, it is now clear that they play active roles in intercellular communication.^{99, 100} Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) have been shown to elicit similar biological effects to MSCs after administration in various preclinical disease models, including models for kidney, cardiac and brain injury.¹⁰¹⁻¹⁰⁶ Moreover, recently, a therapy-refractory graft-versus-host disease patient was successfully treated with allogeneic MSC-EVs. MSC-EVs induced immune suppressive effects *in vitro* and *in vivo* without any other side effects.¹⁰⁷ In addition, application of extracellular vesicles avoids potential concerns associated with administration of living cells such as tumor formation.^{106, 108}

ANIMAL MODEL

To study the effects of intra-uterine inflammation and hypoxia-ischemia on fetal brain injury and development, various animal models have been developed including mice, rats, guinea pigs, rabbits, pigs, and sheep.²⁵ In this thesis, therapeutic strategies for preterm brain injury were investigated in the ovine fetus, since ovine developmental biology closely resembles the human situation. For example, alveolarization of the fetal lungs, formation of intestinal crypts and villi, and white matter myelination predominantly occur *in utero* in both humans and sheep, whereas these processes are postnatal events in other species (i.e. rodents).¹⁰⁹ Furthermore, as in humans, sheep have a gyrencephalic brain compared to rodents that are lissencephalic and the abundance of cerebral white matter and its anatomic similarities to that of the preterm infant make fetal sheep ideal for neuropathological correlation with humans.¹¹⁰ The long gestation of fetal sheep (~147 days) allows selection of an appropriate developmental stage during which brain insults can be induced and evaluated. Additionally, the docile nature of sheep allows for minimal invasive handling for induction of inflammation through intra-amniotic injections of micro-organisms or endotoxins. Also, the relative large size of fetal lambs allows for chronic instrumentation to enable physiological measurements (e.g. blood pressure, oxygenation), electrophysiological recordings, and application of clinically relevant insults (i.e. intra-amniotic injection of infectious agents and global hypoxia-ischemia). Therefore, preterm sheep are considered to be an excellent translational model to study the effects of inflammation on the developing brain.

THE INSTRUMENTED FETAL SHEEP MODEL

All studies comprising global hypoxia-ischemia in this thesis were performed in a translational model of global hypoxia-ischemia in the preterm ovine fetus in which ovine fetuses were equipped with vascular catheters, electrocardiogram and electroencephalogram electrodes and an inflatable vascular occluder around the umbilical cord for remote induction of transient umbilical cord occlusion *in utero* (figure 1).^{45, 88, 111} Chronic instrumentation allows for continuous recordings and off-line analysis of vital physiological parameters such as brain function with EEG, electrophysiological cardiac function with ECG, hemodynamic stability (baroreflex) with blood pressure and ECG, and blood gas analysis.

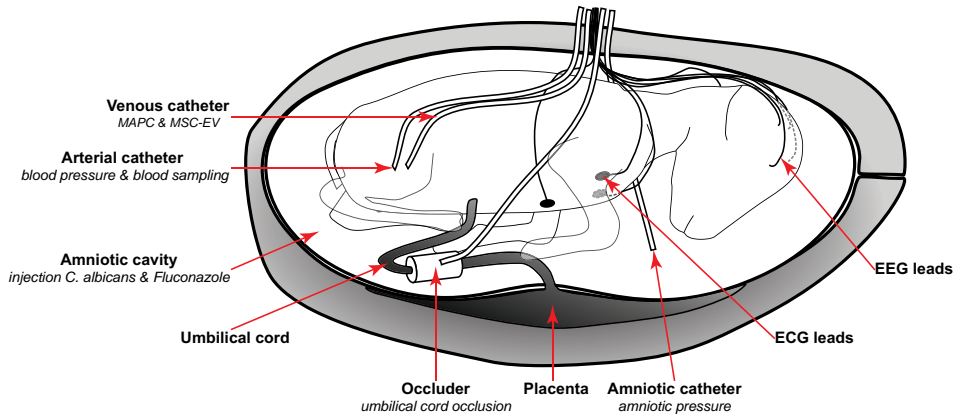


Figure 1. Sheep model of fetal brain injury caused by hypoxia-ischemia or intra-uterine infection.

For experiments comprising global hypoxia-ischemia (i.e. Chapters 2 and 3), ovine fetuses were equipped with electroencephalogram electrodes on the head and electrocardiogram electrodes and thorax. An inflatable vascular occluder was placed around the umbilical for induction of global hypoxia-ischemia. An arterial catheter was placed for blood pressure monitoring and blood sampling. A venous catheter was placed for administration of MAPC cells or MSC-EVs. An additional amniotic catheter was placed for measurement of amniotic pressure and subtracted from the arterial blood pressure to obtain the real blood pressure.

In chapter 4, intra-uterine infection was induced by injecting *C. albicans* directly into the amniotic cavity under ultrasound guidance without surgical intervention and instrumentation. Fluconazole was injected into the amniotic cavity under ultrasound guidance.

OUTLINE OF THE THESIS

The aim of the current thesis was to investigate therapeutic strategies for the prevention of injury of the preterm brain caused by infectious and sterile inflammatory triggers.

In chapters 2 and 3 neuroprotective strategies for sterile inflammation caused by global hypoxia-ischemia are described. Cell-based therapies have proven neuroprotective potential following global hypoxia-ischemia. A major obstacle, however, is the availability of sufficient numbers of stem cells within the limited time in the “window of opportunity”. In chapter 2 we investigated the neuroprotective potential of systemically delivered clinical-grade multipotent adult progenitor cells after global hypoxia-ischemia in the preterm ovine fetus. We hypothesized that systemic administration of a clinical-grade MAPCs product, would attenuate cerebral and peripheral inflammation and prevent structural and functional brain injury after global HI in the preterm ovine fetus.

The promising potential for cell-based therapies as a treatment for preterm brain injury are considered to be primarily driven by paracrine mediators, stored in extracellular vesicles. This suggests that administration of extracellular vesicles rather than intact

stem cells are sufficient to exert therapeutic effects. In **chapter 3**, we therefore investigated the protective effects of systemically administered mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) after hypoxia-ischemia-induced injury of the preterm ovine brain. We hypothesized that systemic administration of MSC-EVs would be neuroprotective in hypoxic-ischemic injury in the preterm brain.

In **chapter 4**, we studied the mechanisms underlying *C. albicans*-induced fetal brain injury. We hypothesized that antenatal exposure to *C. albicans* caused a neuroinflammatory response and subsequent white matter injury, which we tested by exposing fetal sheep to intra-amniotic *C. albicans*. In addition, we tested whether these potential adverse outcomes were improved by *in utero* antifungal treatment with Fluconazole. We therefore further hypothesized that fetal intra-amniotic and intra-peritoneal administration of Fluconazole would reduce the neuroinflammatory response and subsequent white matter injury to the fetal brain.

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2

Clinical-grade Multipotent Adult Progenitor Cells for Hypoxic-Ischemic Injury in the Preterm Brain

Based on:

Clinical-grade Multipotent Adult Progenitor Cells for Hypoxic-Ischemic Injury in the Preterm Brain.

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ABSTRACT

Preterm infants are at risk for hypoxic-ischemic encephalopathy. No therapy exists to treat this brain injury and subsequent long-term sequelae. We have previously shown in a well-established preclinical model of global hypoxia-ischemia (HI) that mesenchymal stromal cells are a promising candidate for the treatment of hypoxic-ischemic brain injury. In the current study we investigated the neuroprotective capacity of multipotent adult progenitor cells (MAPC), which are adherent bone marrow derived cells of an earlier developmental stage than mesenchymal stromal cells and exhibiting more potent anti-inflammatory and regenerative properties.

Instrumented preterm sheep fetuses were subjected to global hypoxia-ischemia by 25 minutes of umbilical cord occlusion at a gestational age of 106 (term ~147) days. During a 7-day reperfusion period, vital parameters (e.g. blood pressure and heart rate; baroreceptor reflex) and (amplitude-integrated) electroencephalogram were recorded. At the end of the experiment, the preterm brain was studied by histology.

Systemic administration of MAPC reduced the number and duration of seizures and prevented decrease in baroreceptor reflex sensitivity after global HI. In addition, MAPC prevented HI-induced microglial proliferation in the preterm brain. These anti-inflammatory effects were associated with MAPC-induced prevention of hypomyelination after global HI. Besides attenuation of the cerebral inflammatory response, our findings showed that MAPC modulated the peripheral splenic inflammatory response, which has been implicated in the etiology of hypoxic-ischemic injury in the preterm brain.

In a preclinical animal model MAPCs improved the functional and structural outcome of the preterm brain after global HI. Future studies should establish the mechanism and long-term therapeutic effects of neuroprotection established by MAPCs in the developing preterm brain exposed to HI. Our study may form the basis for future clinical trials, which will evaluate whether MAPCS therapy is capable of reducing neurological sequelae in preterm infants with hypoxic-ischemic encephalopathy.

INTRODUCTION

Hypoxia-ischemia (HI) in the developing brain is strongly correlated with mortality and neurological morbidity in preterm and full-term infants, resulting in an enormous physical, psychological and economic burden.¹ Unfortunately, no therapeutic cure is available for hypoxic-ischemic brain injury in preterm infants.

In a translational animal model of global HI in the preterm ovine fetus, our group has demonstrated that cell-based therapy may be a promising neuroprotective strategy for preterm neonates suffering from HI-induced brain injury.² We showed that intravenous administration of exogenous mesenchymal stromal cells (MSCs) protected against functional loss and structural injury of the preterm brain.² These neuroprotective effects were largely attributable to attenuation of (neuro) inflammatory processes, as indicated by decreased microglial activation and proliferation in the preterm brain and induction of peripheral T-cell tolerance, which was associated with reduced cerebral infiltration of these immune effector cells.²

Multipotent adult progenitor cells (MAPCs), which are adherent bone marrow derived cells of an earlier developmental stage than MSCs have a high expansion potential and their immunological properties make it possible to use them as a universal allogeneic donor.³⁻⁵ Pre-clinical animal studies have demonstrated that in comparison to MSCs, MAPCs have stronger anti-inflammatory effects and are more potent in promoting endogenous tissue regeneration after ischemic and traumatic injury to the central nervous system (CNS).⁶⁻¹¹ In addition, the smaller size of MAPCs compared to MSCs facilitates passage through the pulmonary capillary bed, which may increase availability of MAPCs in the systemic and cerebral vasculature and thus enhancing their neuroprotective effect.¹²

Based on these superior qualities of MAPCs, we aimed to assess the anti-inflammatory and neuroprotective potential of MAPCs in the preterm brain exposed to global HI. We hypothesized that systemic administration of a clinical-grade MAPC product, would attenuate cerebral and peripheral inflammation and prevent structural and functional brain injury after global HI in the preterm ovine fetus. We tested our hypothesis in a well-established pre-clinical animal model of preterm global HI. In this model, preterm ovine fetuses were exposed to global HI by transient umbilical cord occlusion (UCO) at 0.7 of gestation which is equivalent to 30-32 weeks human gestation¹³, followed by systemic administration of MAPCs during a 7 day reperfusion period.

The anti-inflammatory effects of MAPCs were assessed in the preterm brain by analysis of microglial responses in the hippocampus and subcortical white matter. The spleen was assessed, since previous reports suggest that MAPC-induced alterations in the splenic inflammatory response may be responsible for their neuroprotective effect after brain injury.⁹ Structural effects of systemic MAPC administration were assessed by histological examination of white matter injury. Brain function was studied by analysis

of cortical function by means of electrographic seizure activity and central (brain stem) function by means of baroreceptor reflex sensitivity.

MATERIALS & METHODS

Study approval

The experimental protocol and study design were in line with the institutional guides for animal experiments and were approved by the institutional Animal Ethics Research committee of Maastricht University, the Netherlands.

Randomization and blinding

Thirty-two singleton fetuses of time-mated Texel ewes were randomized by an independent researcher who was not involved in the animal experiments. Randomization resulted in four experimental groups: (1) sham umbilical cord occlusion, saline treatment (sham-SAL n=8), (2) sham umbilical cord occlusion, MAPC treatment (sham-MAPC; n=8), (3) umbilical cord occlusion, saline treatment (HI-SAL; n=8) and (4) umbilical cord occlusion, MAPC treatment (HI-MAPC; n=8) (Figure 1). The investigator performing the (sham) umbilical cord occlusions was blinded to treatment allocation. Tissue sampling and the analyses of brain tissue and electrophysiological data were conducted in a blinded fashion.

Animals and surgery

Singleton fetuses were surgically instrumented at 102 days gestational age (term ~147 days) as described previously.¹⁴ Briefly, fetuses were partially exposed through a midline incision. A polyurethane umbilical vessel catheter (1.2 mm, Covidien, Mansfield, Massachusetts, USA) was placed in the left femoral artery and brachial vein for blood pressure recordings, blood sampling, and administration of MAPC. An additional catheter was placed into the amniotic sac for measurement of amniotic fluid pressure. Three electrocardiogram (ECG) electrodes were placed on the fetal thorax for cardiac monitoring. Two pairs of custom-made shielded silver-tipped electroencephalogram (EEG) electrodes (Cooner wire Co., Chatsworth, CA, USA) were placed bilaterally on the dura over the parasagittal parietal cortex (5 with a subcutaneous silver reference electrode placed in the neck. An inflatable vascular occluder (OC16HD, 16mm, In Vivo Metric, Healdsburg, CA, USA) was placed around the umbilical cord for induction of transient global HI. All fetal leads were exteriorized through the maternal flank. Post-operatively sheep were housed individually with access to water and food *ad libitum*. A period of 4 days post-operative recovery was incorporated before onset of the experiments.

Experimental design

At 106 days gestational age (experimental day 0) fetuses were subjected to 25 minutes of (sham) umbilical cord occlusion by rapidly inflating the occluder with sterile saline of a defined volume known to completely inflate the occluder. An acute drop in heart rate and a gradual decline in blood pressure confirmed complete occlusion of the umbilical cord (Figure 2). Global hypoxia-ischemia was further monitored with subsequent arterial blood gas analysis indicating acidemia, hypoxemia and hypercapnia (data not shown). One hour and 4 days after umbilical cord occlusion and on experimental day 4 fetuses received either an intravenous bolus of MAPCs or an equal volume of vehicle. At the end of the experiment (experimental day 7), both ewe and fetus were euthanized by administration of pentobarbital (200 mg/kg).

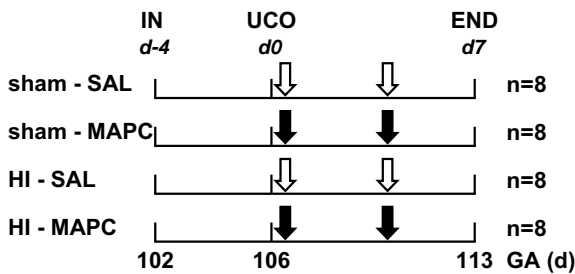


Figure 1. Study design.

Fetuses were instrumented at a gestational age (GA) of 102 d. After a recovery period of 4 d fetuses were subjected to 25 min of umbilical cord occlusion (UCO) or sham. One hour and 4 days after UCO or sham, fetuses received either intravenous MAPCs (10 million cells, closed arrow) or saline 0.9% (open arrow). After a 7 d reperfusion period brain tissue was collected. Abbreviations: in = instrumentation, HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells.

Data acquisition and analysis

Physiological data was sampled as described previously on a custom-made MPAQ unit (Maastricht-Programmable AcQuisition system, Maastricht Instruments BV, Maastricht, the Netherlands) with IDEEQ software (Maastricht Instruments BV).¹⁴ In short, EEG data were sampled at 1000 Hz and stored on hard-disk for off-line analysis. EEG data were filtered using a 0.5-30 Hz 4th order Butterworth band-pass filter. EEG signal with an amplitude > 1000 μ V was considered an artifact and removed from analysis (<1% of data). After filtering, the raw EEG signals of the central and posterior channels were converted into amplitude-integrated EEG (aEEG) traces, using an (Matlab[®] based) algorithm similar to the clinical EEG NicoletOne[™] device (Viasys Healthcare, Conshohocken, PA, USA), as previously described.^{2, 15-18} EEG seizure activity was annotated manually using aEEG/EEG traces and the total number and length of seizures

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was subsequently calculated. A neonatologist experienced in aEEG interpretation and blinded to treatment allocation, performed annotation.

ECG and blood pressure data were processed off-line using MATLAB R2013a (The MathWorks, Inc., Natick, MA). The ECG recordings were used to determine R-R intervals. Blood pressure measurements were corrected for amniotic pressure and used to determine the mean arterial blood pressure in each pressure wave. As previously reported, heart rate and heart rate variability were assessed in 192 s segment by calculating the mean R-R interval and standard deviation (SD-RR) respectively.^{19, 20} Likewise, mean systolic blood pressure and blood pressure variability was assessed by calculating the mean SBP and standard deviation (SD-SBP).¹⁹ As a time series estimate of baroreceptor reflex sensitivity, we calculated the ratio between SD-RR and SD-SBP.^{19, 21, 22}

MAPCs

Multipotent Adult Progenitor Cells were provided by Athersys, Inc. (Cleveland OH, USA) and stored in liquid nitrogen. Cells were prepared under good manufacturing practice (GMP) and genomic and epigenetic stability of the cells were confirmed as previously described.^{5, 23} Flow cytometric analysis of cell-surface proteins demonstrated that the cells were CD29, CD90, CD44 positive, and MHC class I low, and were negative for MHC class II, CD45, CD106, and the costimulatory molecules CD80 and CD86. Cells were able to differentiate into mesenchymal cell types (adipocytes, osteoblasts, chondrocytes, and smooth muscle cells), but also towards endothelium.⁴ Prior to injection, MAPCs were thawed and washed twice and suspended in phosphate buffered saline (PBS) at a concentration of 10×10^6 cells/mL. MAPCs were administered intravenously 1 hour and 4 days after (sham) UCO. Fetuses received two doses of 10×10^6 cells in 1mL PBS.

Immunohistochemistry in the brain

After 3 months of submersion fixation in 4% paraformaldehyde, the right cerebral hemisphere was embedded in gelatin and serial coronal sections (50 μ m) were cut on a Leica VT 1200S vibrating microtome (Leica Biosystems, Nussloch, Germany). Free floating sections at the level of mid-thalamus and posterior hippocampus were stained with a rabbit anti-ionized calcium binding adaptor molecule 1 (IBA-1) antibody (Wako Pure Chemical Industries, Osaka, Japan) for microglia or a rat anti-myelin basic protein (MBP) antibody (Merck Millipore, Billerica, MA, USA) for myelin sheaths and myelin producing (mature) oligodendrocytes, and cleaved Caspase-3 for apoptosis (Cell Signaling Technology, Boston, USA) as previously described.^{14, 24}

Briefly, endogenous peroxidase-activity was blocked by incubation with 0.3% H_2O_2 in Tris Buffered Saline (TBS, pH 7.6). Free floating sections were incubated overnight at 4°C with the diluted primary antibody (1:1000 anti-IBA-1, 1:2000 MBP, 1:800 Caspase-3) followed by

incubation with the appropriate secondary antibodies. The immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, CA, USA) followed by a nickel sulfate-diaminobenzidine (IBA-1) or 3,3'-diaminobenzidine staining (MBP, Caspase-3). Sections were mounted on gelatin-coated glass slides, air-dried, dehydrated in ascending ethanol concentrations and coverslipped with PerTex.

Analysis of immunohistochemistry

Immunohistochemical stainings were analyzed as previously described, for the analysis of IBA-1 and MBP immunoreactivity¹⁴ and densities of Caspase-3 positive cells. For the analysis of IBA-1 and MBP immunoreactivity (IR), digital images of the subcortical white matter (SCWM) (100x magnification) and hippocampus (20x magnification; only IBA-1) were acquired using an Olympus AX-70 microscope (Olympus, Tokyo, Japan) equipped with a black and white digital camera. In the hippocampus, area fraction of IBA-1 immunoreactivity was assessed in one digital image per section by delineating the hippocampus and determining the areal fraction of IBA-1 immunoreactivity expressed as a percentage of total hippocampal area with a standard threshold using Leica Qwin Pro V 3.5.1 software (Leica, Rijswijk, the Netherlands). In the SCWM, the area fractions of IBA-1 and MBP immunoreactivity were determined in five adjacent 100x digital images obtained in standardized locations within the SCWM of each section. The results of five images per section were averaged to obtain the areal fractions of IBA-1 and MBP immunoreactivity within the SCWM for each section.

For analysis of cleaved Caspase-3, 5 adjacent digital images (100x magnification) of the SCWM were obtained in standardized locations for each section. Numbers of Caspase-3 positive cells were determined with Leica Qwin Pro V 3.5.1 software, and averaged to obtain cell numbers for Caspase-3 per section.

RNA extraction and quantitative real-time PCR

For real-time (RT) quantitative polymerase chain reactions (qPCR), total RNA was extracted from spleen using the SV Total RNA isolation system (Z3100; Promega, Madison, WI) according to the manufacturer's recommendations. RT qPCR reactions were performed in duplicate with the SensiMix SYBR No-ROX kit (QT650-02; Bioline Reagents Ltd) in a LightCycler 480 Instrument with ovine specific primers for interleukin IL-10 (5'-CATGGGCC TGACATCAAGGA-3' (sense), 5'-CGGAGGGTCTTCAGCTTCTC-3' (antisense), tumor necrosis factor alpha (TNF α) (5'-GCCGGAATACCTGGACTATGC-3' (sense), 5'-CAGGGCGATGATCCCAA GTAG-3' (antisense), and interferon gamma (IFN γ) (5'-TCAAGCAAGACATGTTTCAGAAGTTCT-3' (sense), 5'-CCGGAATTTGAATCAGCCTTTTGAA-3' (antisense). RT qPCR results were normalized to the housekeeping gene ovine 40S ribosomal protein S15 (ovRPS15) (5'-CGAGATGGTGGGCAGCAT-3' (sense), 5'-GCTTGATTTC CACCTGGTTGA-3' (antisense)).

MAPC detection

Detection of MAPC was performed with a PCR for human β -2 microglobulin.² Genomic DNA was isolated from snap frozen brain, spleen and lung tissues using the Wizard Genomic Purification Kit (Promega, Leiden, The Netherlands) according to the manufacturer's recommendations. PCR reactions of genomic DNA were performed for β -2 microglobulin followed by a nested PCR for β -2 microglobulin on the amplification product of the first PCR.

The amplified products were separated on ethidium bromide-stained 2% agarose gels and captured using the Imagemaster® VDS equipped with a CCD camera (GE Healthcare Life Sciences (Pharmacia Biotech), Uppsala, Sweden). Serial dilutions of MAPCs were used as references.

Primers used for the amplification of β -2 microglobulin were 5'- GTGTCTGGGTTTCAT CAATC-3' (sense), 5'- GGCAGGCATACTCATCTTTT-3' (antisense), 5'- TGGGTTTCATCAA TCCGACAT-3' (nested sense) and 5'- CTCATCTTTTTCAGTGGGGT-3' (nested antisense).

Statistics

Summary statistics of animal characteristics and all outcome variables are shown as means with 95% Confidence Interval (CI). Groups' comparisons of all outcome parameters were drawn with analysis of variance (ANOVA) or with random intercept models in case of repeated measurements per animal (e.g. different sections per brain). HI (sham vs. HI) and treatment (saline vs. MAPC) were the fixed effects. For random intercept models, animals constituted additionally the random effect. Variables, whose distributions were positively skewed, were log-transformed previous to statistical testing. To facilitate interpretation, averages on the log scale were back transformed to the original scale (antilog) and were presented as geometric means and corresponding 95% CIs.

Seizure data, measured over time, showed pronounced right-skewness (caused by the absence of seizures in non-hypoxic conditions) that could not be remedied by log transformation. Hence, for these variables, pair-wise groups' comparisons were performed with nonparametric Mann-Whitney tests, per individual time-point. They are presented as medians and corresponding interquartile range (IQR). A False Discovery Rate (FDR) of 5% was used for multiple testing correction. Groups' differences with FDR corrected $P < 0.05$ were considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics Version 20.0 (IBM Corp., Armonk, NY, USA).

Statistical analysis of baroreflex data was performed using a Bayesian multilevel model²⁵ to estimate the daily effects of global HI and MAPC treatment under hypoxic and sham conditions on the baroreceptor reflex sensitivity. This model corrects for repeated measurements and takes into account variance between and within subjects.²⁵

Statistical time series analysis was conducted using the Stan for R package (version 2.6.0) in R (version 3.1.1).

RESULTS

Animal characteristics

To test the neuroprotective potential of clinical-grade MAPCs, we randomized 32 preterm sheep fetuses in four different experimental groups (Figure 1). After instrumentation and a recovery period of four days, animals were subjected to 25 min of (sham) umbilical cord occlusion (UCO) to induce global hypoxia-ischemia (HI). MAPCs were administered 1 hour and 4 days after UCO (Figure 1). Fetal body weight and gestational age at did not differ significantly between the four experimental groups (Table 1). In line with previous reports^{2, 14, 15}, the fetal response to hypoxia-ischemia was comparable in all animals exposed to UCO (Figure 2), indicating that the injury group (HI-SAL) and intervention group (HI-MAPC) were exposed to a similar degree of global hypoxia-ischemia.

Table 1. Animal characteristics.

	sham-SAL	sham-MAPC	HI-SAL	HI-MAPC
GA at UCO (d)	105.2 (105.0 ; 105.5)	106.0 (105.3 ; 106.7)	105.5 (105.1 ; 106.0)	105.2 (104.9 ; 105.8)
BW (g)	1690 (1516 ; 1865)	1532 (1245 ; 1820)	1542 (1362 ; 1721)	1559 (1340 ; 1779)
Brain weight (g/kg BW)	17.58 (16.04 ; 19.12)	17.39 (14.72 ; 20.05)	15.97 (14.43 ; 17.51)*	16.08 (14.37 ; 17.80)

Fetuses were subjected to umbilical cord occlusion (UCO) at a comparable gestational age (GA). Fetal body weight (BW) did not differ between experimental groups. Brain weight corrected for bodyweight was significantly reduced following global HI. MAPC treatment did not prevent loss of cerebral weight. Means \pm 95% CI are shown. Groups' comparisons were drawn with ANOVA (sham-SAL n=8, sham-MAPC n=8, HI-SAL n=8, HI-MAPC n=8) * $P \leq 0.05$. HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells.

MAPCs reduced the cerebral inflammatory response after global HI

The cerebral inflammatory response was studied by assessing microglial proliferation in the subcortical white matter and hippocampus using ionized calcium binding adaptor molecule 1 (IBA-1), which is a highly specific marker for resting and activated microglia in sheep.¹⁴ Global HI resulted in a significant (sham-SAL vs. HI-SAL; $P < 0.001$) increase of IBA-1 immunoreactivity in the SCWM and hippocampus, indicating profound microglial proliferation in these regions (Figure 3A-F). MAPCs significantly (HI-SAL vs. HI-MAPC; $P = 0.043$) reduced IBA-1 immunoreactivity in the SCWM (Figure 3A-C). MAPCs did not

reduce IBA-1 immunoreactivity in the hippocampus (HI-SAL vs. HI-MAPC; $P=0.881$) (Figure 3D-F). No differences in IBA-1 immunoreactivity were observed between saline or MAPC treated sham operated animals in the SCWM and hippocampus.

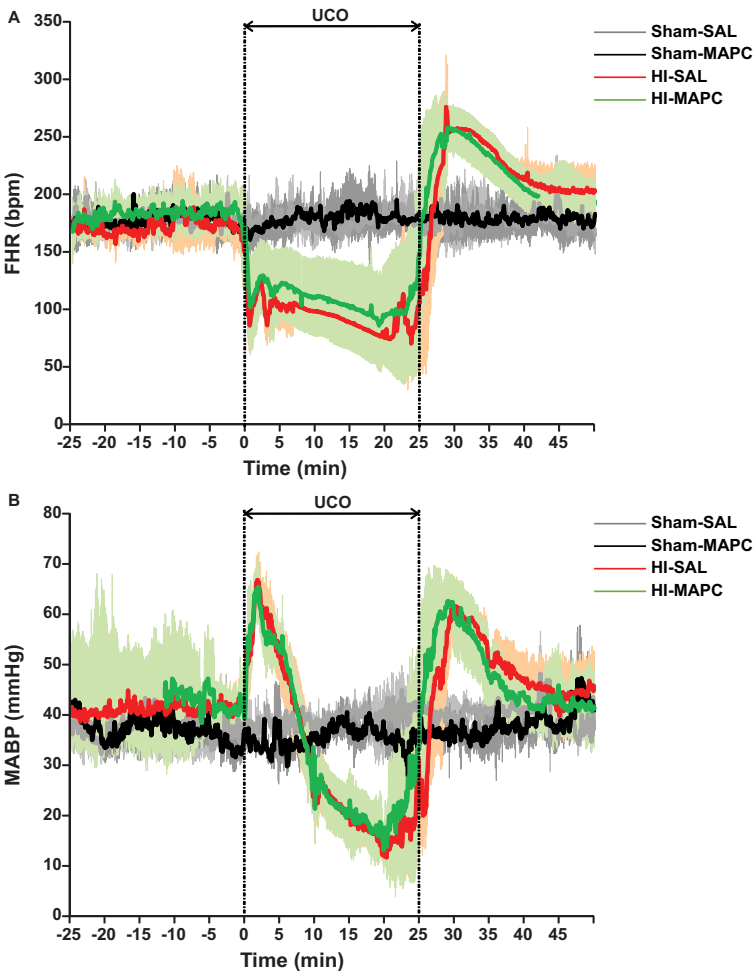


Figure 2. Reproducibility of 25 min umbilical cord occlusion (UCO) as evidenced by comparable vital parameters and blood gases in animals exposed to global HI. (A) Fetal heart rate (FHR) and (B) fetal mean arterial blood pressure (MABP) measurements indicated that all animals exposed to global HI experienced the same degree of bradycardia and hypotension, respectively; means (thick line) \pm SD (shaded areas) of $n=8$ animals per experimental group are shown. HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells, min = minutes

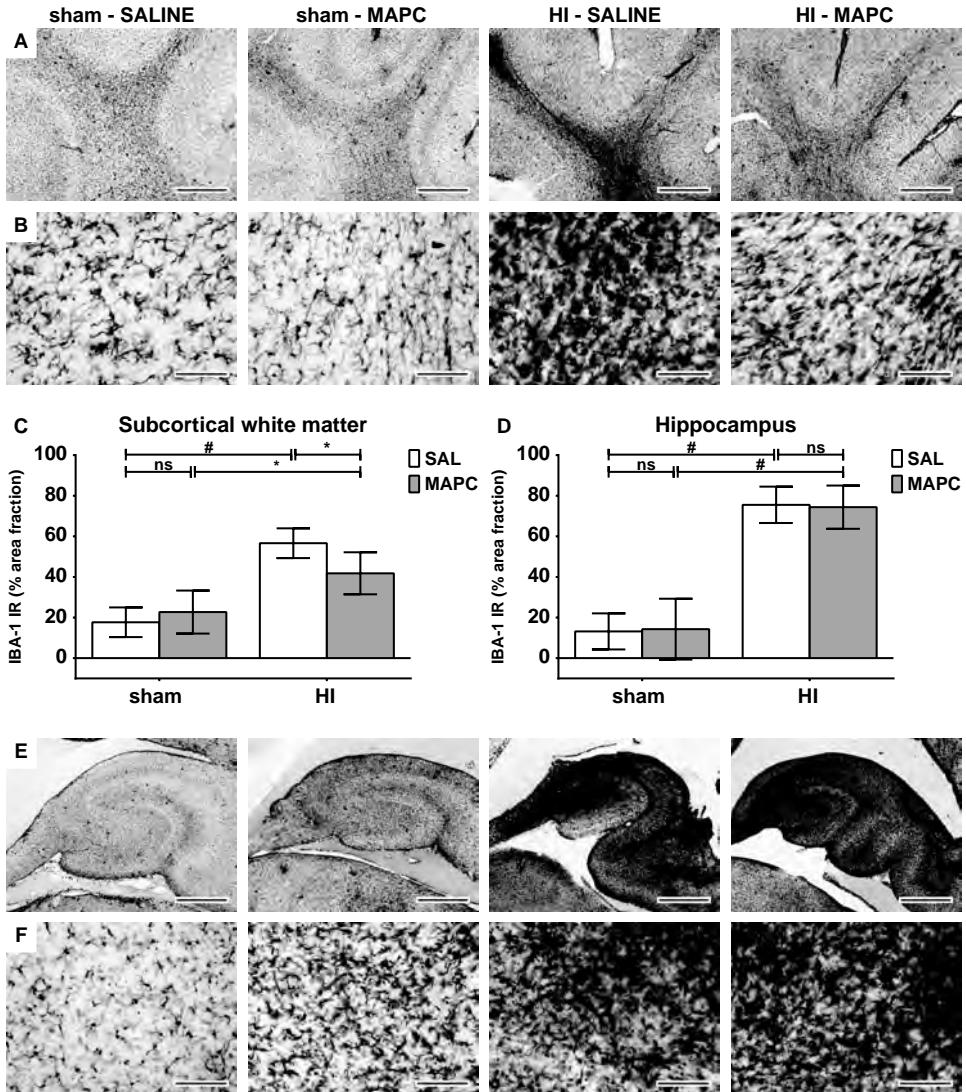


Figure 3. MAPCs reduced cerebral inflammation in the subcortical white matter (SCWM), but not in the hippocampus. (A-B)

Immunohistochemical IBA-1 staining in the SCWM at 20x (A) and 200x (B) magnification of the four experimental groups (sham-SAL n=8, sham-MAPC n=5, HI-SAL n=8, HI-MAPC n=5). Global HI induced a profound increase of IBA-1 immunoreactivity and amoeboid morphology, which was significantly reduced by MAPCs in the SCWM. (C-D) Graphical presentation of area fraction of IBA-1 immunoreactivity in SCWM and hippocampus. Geometric means \pm 95% CI and levels of significance are depicted, which were calculated by the random intercept model with all repeated measures (i.e. brain sections) per animal. (E-F) Immunohistochemical IBA-1 staining in the hippocampus at 20x (E) and 200x (F) magnification of the four experimental groups. IBA-1 IR was greatly increased and accompanied with amoeboid morphology following global HI, which remained unaffected after MAPC treatment. * $P \leq 0.05$, § $P \leq 0.01$, # $P \leq 0.001$. IBA-1 = ionized calcium binding adaptor molecule 1, HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells, IR = immunoreactivity. Scale bars: (A-E) 1 mm, (B-F) 100 μ m.

MAPCs prevented HI-induced white matter injury

White matter injury was studied by assessing myelin basic protein (MBP) immunoreactivity in the SCWM. Global HI significantly (sham-SAL vs. HI-SAL; $P<0.001$) reduced MBP immunoreactivity in the SCWM, indicating HI-induced hypomyelination in this region (Figure 4A-C). MAPCs significantly increased MBP immunoreactivity (HI-SAL vs. HI-MAPC; $P=0.016$). No differences in MBP immunoreactivity in the SCWM were observed between sham animals treated with saline or MAPCs (Figure 4A-C). We determined brain weight as a surrogate for neuronal injury (table 1). Brain weight was significantly reduced after global HI (sham-SAL vs. HI-SAL; $P=0.046$). MAPCs did not prevent loss of brain weight (HI-SAL vs. HI-MAPC; $P=0.429$).

Apoptotic cell death was evaluated in the SCWM by assessment of the number of cleaved Caspase-3 positive cells (Figure 4D). No differences in numbers Caspase-3 positive cells were found 7 days after global HI (sham-SAL vs. HI-SAL; $P=0.135$). The density of apoptotic cells remained unaffected by systemic administration of MAPCs (HI-SAL vs. HI-MAPC; $P=0.881$).

We did not detect MAPCs in brain, spleen or lung tissue 7 days after global HI (i.e. 3 days after the second dose of MAPCs) using PCR for human-specific β -2 microglobulin (data not shown).

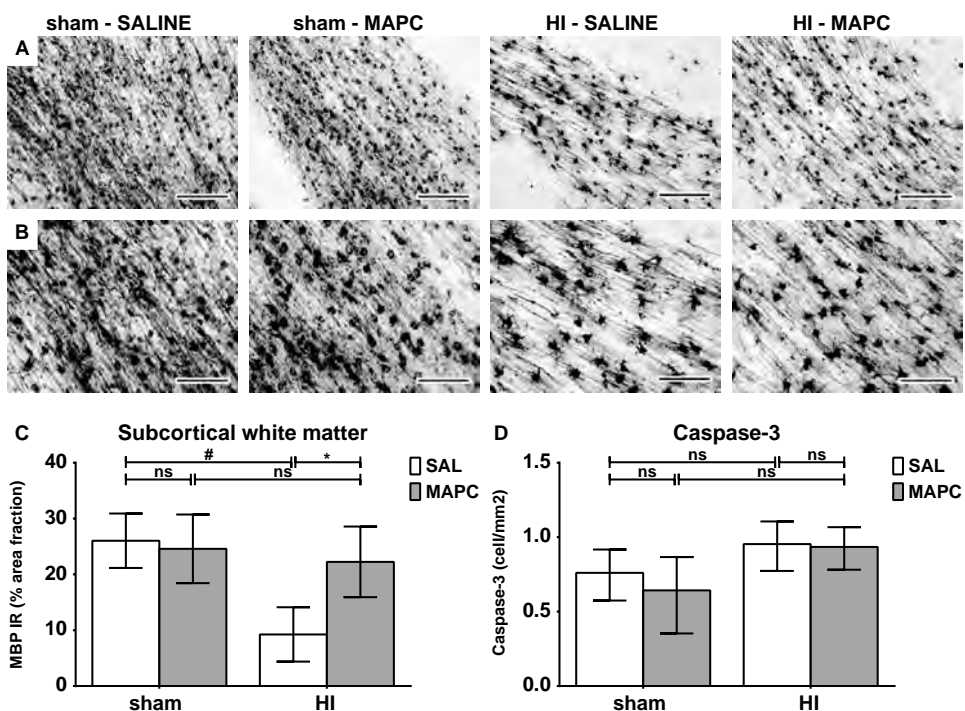


Figure 4. MAPCs reduced white matter injury after global HI.

(A-B) Immunohistochemical MBP staining in the SCWM of the four experimental groups at (A) 100x and (B) 200x magnification. (C) MBP immunoreactivity in SCWM. Global HI induced marked hypomyelination in the SCWM. MAPCs significantly prevented the decrease in MBP reactivity after global HI. The area fraction of MBP was similar in sham conditions. Geometric means \pm 95% CI and levels of significance are depicted, which were calculated by the random intercept model with all repeated measures (i.e. brain sections) per animal (sham-SAL n=8, sham-MAPC n=5, HI-SAL n=8, HI-MAPC n=5). (D) Numbers of Caspase-3 positive cells in the four experimental groups; geometric means \pm 95% CI and levels of significance are depicted, which were calculated with ANOVA (sham-SAL n=8, sham-MAPC n=8, HI-SAL n=8, HI-MAPC n=8). * $P \leq 0.05$, § $P \leq 0.01$, # $P \leq 0.001$. MBP = myelin basic protein 1, HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells, IR = immunoreactivity. Scale bars: (A) 200 μ m, (B) 100 μ m.

MAPCs prevented splenic involution and modulated the peripheral inflammatory response

Splenic weight was assessed, since activation of the splenic inflammatory response was previously shown to be characterized by mobilization of effector immune cells affecting volume and weight of the spleen.¹⁴ Global HI induced splenic involution in saline treated animals (sham-SAL vs. HI-SAL, $P=0.022$) (Figure 5A). MAPCs significantly prevented splenic involution after global HI (HI-SAL vs. HI-MAPC, $P=0.032$). The splenic cytokine profile was studied since previous findings indicated that MAPCs established neuroprotection by modulating the splenic inflammatory response (Figure 5B-D). Splenic IL-10 mRNA levels were significantly increased after global HI (sham-SAL vs. HI-SAL, $P=0.031$). MAPCs significantly reduced IL-10 mRNA levels in the spleen after global HI (HI-SAL vs. HI-MAPC, $P=0.042$) (Figure 5B). IFN γ mRNA levels were significantly increased following global HI (sham-SAL vs. HI-SAL; $P=0.022$). Systemic administration of MAPCs tended to reduce IFN γ mRNA levels, but statistical significance was not reached (HI-SAL vs. HI-MAPC; $P=0.078$) (Figure 5C). Global HI tended to increase splenic TNF α mRNA levels (sham-SAL vs. HI-SAL; $P=0.078$). However, MAPC treatment significantly reduced TNF α mRNA levels (HI-SAL vs. HI-MAPC; $P<0.001$) (Figure 5D).

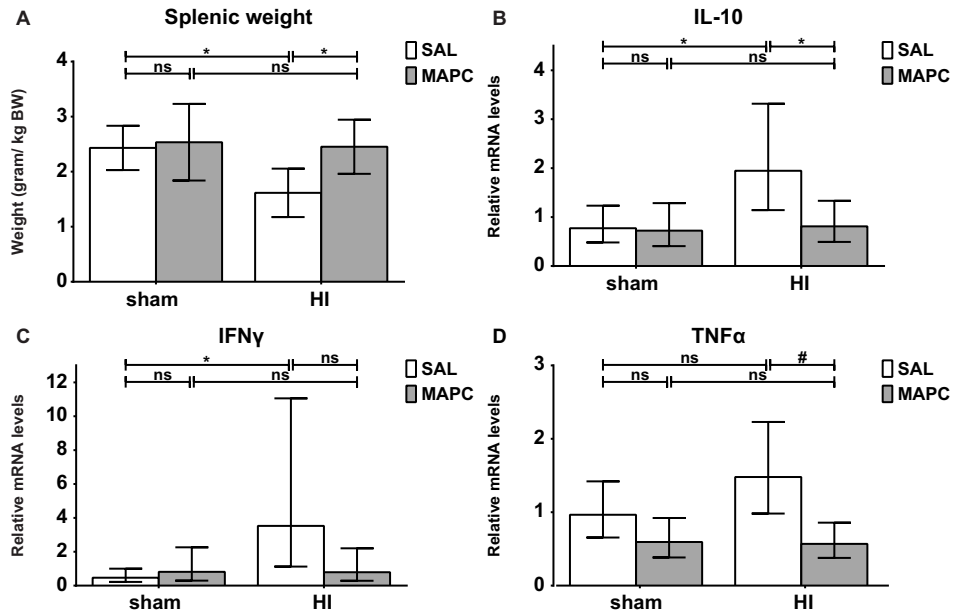


Figure 5. MAPCs prevented splenic involution and modulated the peripheral inflammatory response. (A) Splenic weight corrected for body weight (BW). Global HI induced significant splenic involution, which was significantly prevented by MAPCs; means \pm 95% CI and levels of significance are depicted, which were calculated with ANOVA (sham-SAL n=8, sham-MAPC n=8, HI-SAL n=8, HI-MAPC n=8). (B) IL-10 mRNA levels were significantly increased in the spleen 7 days after global HI. MAPCs significantly attenuated the HI-induced IL-10 response in the spleen. (C) Splenic IFN γ mRNA levels were significantly increased 7 days following global HI. MAPC treatment tended to reduce IFN γ mRNA levels. However, statistical significance was not reached. (D) TNF α mRNA levels showed a trend to increase following global HI, which was significantly prevented by MAPCs; geometric means \pm 95% CI and levels of significance are depicted, which were calculated with ANOVA (sham-SAL n=8, sham-MAPC n=6, HI-SAL n=8, HI-MAPC n=8). * $P \leq 0.05$, § $P \leq 0.01$, # $P \leq 0.001$. HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells, IFN γ = interferon gamma, TNF α = tumor necrosis factor alpha.

MAPCs reduced seizure burden after global HI

Electrographic seizure activity was assessed to determine the effect of MAPCs on cortical function after global HI (Figure 6A-B). Global HI induced a significant increase in total number (Figure 6A) and duration (Figure 6B) of seizures after global HI (sham-SAL vs. HI-SAL, significant in all time points). Seizure burden peaked within the first 48 hours following global HI with a second small rise in activity around experimental day 4 and 5 (Figure 6A-B). MAPCs reduced seizure burden throughout the complete reperfusion period (HI-SAL vs. HI-MAPCS, significant from experimental day 2 onwards). Cumulative number of seizures in the complete reperfusion study was significantly increased after global HI (sham-SAL vs. HI-SAL, $P=0.016$) and significantly reduced by MAPCs (HI-SAL vs. HI-MAPC, $P=0.022$) (Figure 6A). Similarly, cumulative duration (sec) of seizures was

significantly increased after global HI (sham-SAL v. HI-SAL, $P=0.015$) and significantly reduced by MAPCs (HI-SAL vs. HI-MAPC, $P=0.043$) (Figure 6B). No electrographic seizure activity was detected under sham conditions.

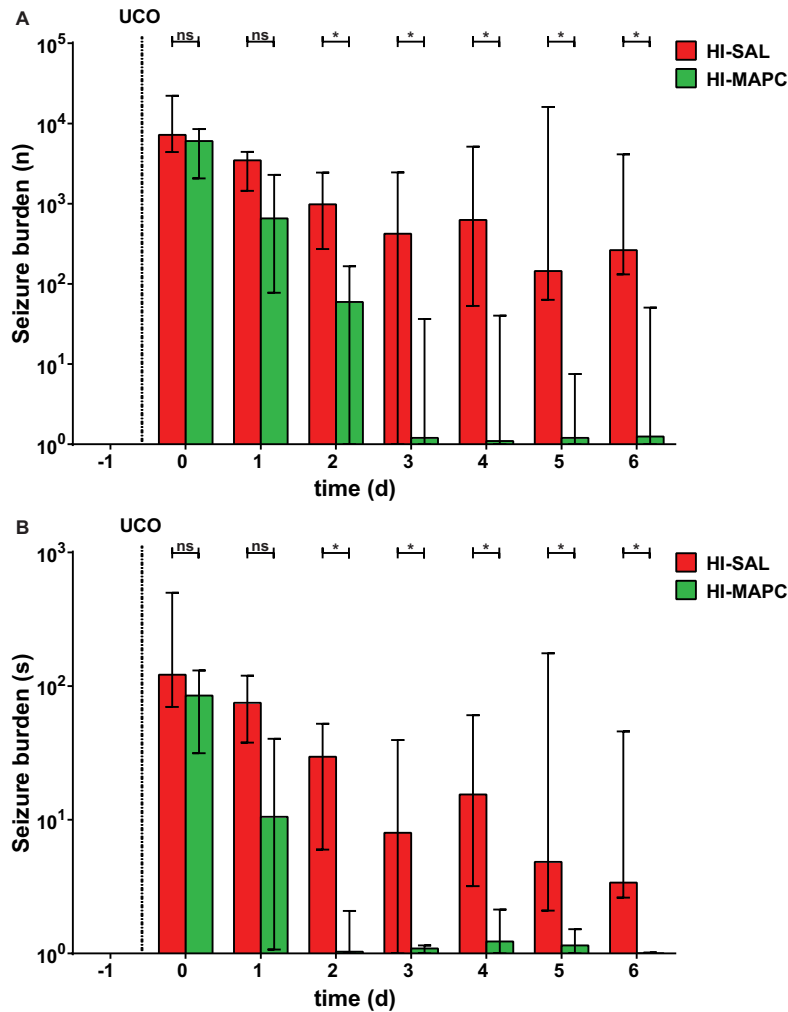


Figure 6. MAPCs induced functional neuroprotection after global HI.

Global HI caused a significant seizure burden indicated by an increased (A) total number and (B) duration of seizures compared to controls. Administration of MAPCs significantly reduced electrographic seizure number and duration. Medians \pm interquartile ranges (IQR) and levels of significance of the treatment effect (HI-SAL vs. HI-MAPC) are depicted, which were calculated by Mann-Whitney test (HI-SAL $n=8$, HI-MAPC $n=8$). No electrographic seizure activity was detected under sham conditions. For readability purposes these sham groups (sham-SAL $n=8$ and sham-MAPC $n=6$) are not shown. * $P \leq 0.05$, § $P \leq 0.01$, # $P \leq 0.001$. HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells

MAPCs prevented loss of baroreceptor reflex sensitivity after global HI

Baroreceptor reflex sensitivity was analyzed to study the effect of MAPC treatment on deeper brain functions situated in the brain stem (Figure 7). Global HI increasingly compromised baroreceptor reflex sensitivity over time (sham-SAL vs. HI-SAL, significant for all time points with the exception of experimental day 1). MAPCs significantly prevented the loss of baroreceptor reflex sensitivity (HI-SAL vs. HI-MAPC, significant on all time points).

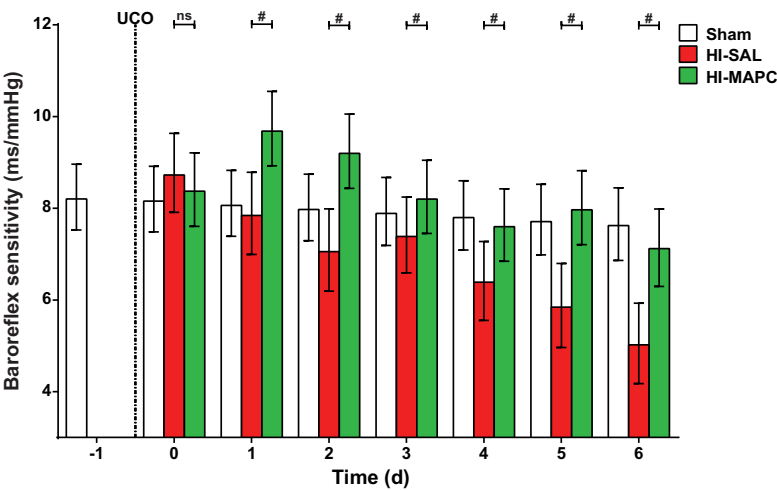


Figure 7. MAPCs prevented loss of baroreceptor reflex sensitivity.

Global HI caused a significant gradual decline of baroreceptor reflex sensitivity over time, which was prevented by MAPC treatment; means \pm 95% CI and levels of significance of the treatment effect (HI-SAL vs. HI-MAPC) are depicted, which were calculated by the Bayesian multi-level model. There were no differences in baroreceptor reflex sensitivity between the sham-SAL and sham-MAPC groups. For readability purposes all sham treated animals (sham-SAL n=8 and sham-MAPC n=6) were grouped and depicted as one sham group. HI-SAL n=8, HI-MAPC n=8. * $P \leq 0.05$, § $P \leq 0.01$, # $P \leq 0.001$. HI = hypoxia-ischemia, SAL = saline, MAPC = multipotent adult progenitor cells.

DISCUSSION

Hypoxic-ischemic brain injury is common in preterm infants. To date no treatment options are available to reduce mortality and morbidity associated with brain injury in preterm infants. Our group has previously shown in a well-established pre-clinical model of global hypoxic-ischemia (HI) that mesenchymal stromal cells are a promising candidate for the treatment of hypoxic-ischemic brain injury.² In the current study we investigated the neuroprotective capacity of multipotent adult progenitor cells (MAPCs), which are a subset of bone marrow-derived MSCs with more potent anti-

inflammatory and regenerative properties.^{3, 4, 6-10} We showed that intravenous administration of MAPCs prevented functional and structural injury in the preterm brain and modulated the cerebral and peripheral inflammatory response after global HI.

We administered MAPCs 1 hour and 4 days after global HI. With the first dose we aimed to dampen the acute cerebral and peripheral inflammatory response, which has been shown to evolve early after global hypoxia-ischemia.² A pilot study in this model showed a secondary peak in seizure burden 4-5 days after global HI (unpublished data). Therefore, the second dose of MAPCs was administered 4 days after global HI aimed at preventing secondary functional injury.

Our findings showed that MAPCs 1 hour and 4 days after global HI reduced seizure burden after global HI throughout the study period of 7 days. This finding is clinically highly relevant, since several studies have shown that seizures in term and preterm neonatal HIE are associated with adverse neurodevelopmental outcome.²⁶⁻²⁸ In our model we detected seizure activity with amplitude-integrated EEG (aEEG), which is clinically used to monitor neonatal brain function and has a high sensitivity and specificity in predicting neurodevelopmental outcomes in neonates with HI brain injury.²⁹ Recorded seizure activity in our study was most likely located in the cortical layers. However, since the electrodes were placed directly on the dura, we may have recorded seizures or electrophysiological responses originating from deeper regions underlying the cortex (i.e. thalamus and hippocampus). In addition to cortical grey matter protection, the reduced seizure activity may therefore also represent protection of deeper brain structures. We therefore assessed the baroreceptor reflex as a function of the brain stem. This is clinically of importance since the baroreflex is a vital part of the cardiovascular auto regulatory system in preterm infants, which secures adequate perfusion of the preterm brain.²² The cerebrovascular system is still immature in prematurity making the preterm brain extremely vulnerable to fluctuations in blood pressure caused by HI (or other adverse events such as infection).³⁰ Previously, we demonstrated that global HI disturbed normal development of the baroreflex, which resulted in impairment of heart rate mediated blood pressure control.²² The fact that MAPC treatment at 1 h and 4 days improved baroreceptor reflex sensitivity indicated that the therapeutic effects reach as far as the highly conserved central brain functions in the brainstem and supports the concept of MAPC-induced protection against grey matter injury in the hypoxic-ischemic preterm brain. The protection of grey matter by MAPCs after global HI may be explained by findings of previous reports demonstrating that MAPCs improved neurogenesis and stimulated neurite outgrowth and synaptogenesis.^{7, 31, 32}

Density of apoptotic cells in the subcortical white matter was neither affected by global HI nor by MAPC treatment in this study. The fact that we did not detect significant changes in cell death is most likely explained by timing of assessment at 7 days after global HI, while apoptotic cell death peaks earlier after cerebral hypoxia-ischemia.

Further studies on the dynamics of cell death in the preterm brain after global HI are needed to determine the therapeutic effect of MAPCs on neuronal loss.

MAPCs have been previously shown to have potent anti-inflammatory properties in pre-clinical studies of traumatic and ischemic injury of the central nervous system.⁶⁻¹¹ Our study confirmed and extended these findings in a translational model of hypoxic-ischemic injury in the preterm brain by showing that MAPCs reduced the neuroinflammatory response in the subcortical white matter (SCWM), as evidenced by reduced microglial proliferation. We and others have shown that the microglial response is a reliable marker for the cerebral inflammatory response that plays a pivotal role in the etiology of hypoxic ischemic encephalopathy in the preterm brain.^{14, 33} Although the size of the treatment effect was modest, reduction of the microglial response was associated with functional and structural improvement, indicating the biological relevance of modulating the cerebral inflammatory response mediated by microglia. We postulate that the observed MAPC-induced reduction of cerebral inflammation after global HI may be explained by two mechanisms; firstly, MAPCs have been shown to induce a microglial type switch from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype, which is associated with reduced inflammation in the CNS.⁸ Secondly, MAPCs may have reduced glutamate excitotoxicity in a similar fashion as MSCs, which were shown previously to inhibit glutamate receptor expression and function, thereby inhibiting microglial activation³⁴. However, MAPCs did not attenuate the inflammatory response in the hippocampus. These spatial differences in anti-inflammatory effects are in line with previous data showing that the hippocampus is more severely affected by hypoxia-ischemia and therefore injury may be irreversible.^{2, 14, 15}

White matter injury is the clinical hallmark of HI brain injury in the preterm brain.³⁵ Consistent with earlier studies^{2, 14, 15}, we demonstrated that global hypoxia-ischemia induced marked hypomyelination of the preterm brain, which was prevented by intravenous administration of MAPCs. Microglial type switch and subsequent reduction of cerebral inflammation has been previously associated with remyelination and may therefore explain the observed reduction of white matter injury.^{36, 37}

Besides attenuation of the cerebral inflammatory response, our findings indicated that MAPCs modulated the peripheral inflammatory response, which has been implicated in the etiology of hypoxic-ischemic injury in the preterm brain.^{14, 38-40} Our results showed that MAPCs prevented splenic involution. We have previously shown in the current model that splenic involution after global HI was caused by efflux of immune effector cells.¹⁴ Furthermore, our findings demonstrated that intravenous administration of MSCs induced splenic T-cell tolerance.² The observed unresponsiveness of splenic T-cells was associated with reduced systemic mobilization of immune effector cells and concomitant reduction of brain infiltrating immune cells resulting in less severe inflammation in the preterm brain after global HI.² Based on these findings, we

postulate that MAPCs reduced the mobilization of immune effector cells from the spleen resulting in less extensive invasion of these injurious cells into the brain. This concept is supported by previous findings showing a spleen-mediated neuroprotective effect of MAPCs in traumatic CNS injury.^{9, 11} Recently, it was demonstrated that splenic IFN γ plays a key role in injurious signaling to the ischemic brain.⁴¹ Functional elimination by splenectomy or administration of IFN γ neutralizing antibodies reduced brain injury after stroke.⁴² In line with these findings, reduction of cerebral inflammation (i.e. microglial proliferation) by MAPCs was associated with decreased splenic IFN γ levels. To determine whether IFN γ from splenic cells is involved in preterm brain injury after global HI, temporal dynamics of splenic IFN γ release needs to be elucidated and its functional role to be determined.

We cannot confirm the IL-10 mediated therapeutic effect of MAPCs inducing increased splenic and systemic numbers of protective regulatory T-cells (Tregs) in adult animals.⁹ In our study splenic IL-10 mRNA levels were not altered by MAPCs in sham conditions. Instead, we found a marked increase of IL-10 mRNA under hypoxic-ischemic conditions, which was attenuated by administration of MAPCs. We postulate that the observed increase of splenic IL-10 mRNA after global HI is part of an endogenous regenerative response after injury. MAPCs may have reduced the injurious load eliciting such a response resulting in reduced IL-10 levels at the time of assessment (7 days after global HI). This hypothesis is supported by previous reports showing activation of endogenous repair processes involving IL-10 mediated Treg mobilization after cerebral ischemia.^{39, 40, 43} Furthermore, early administration of MSCs was shown to prevent progression of hypoxic-ischemic injury.² These findings suggest that early administration of MAPCs prevents injury before endogenous repair mechanisms, such as IL-10 mediated Treg mobilization, are activated.

In line with previous studies reporting the clearance of MAPCs within 48-72 hours^{10, 31}, we did not detect MAPCs in brain, spleen or lung tissue 7 days after global HI (i.e. 3 days after the second dose of MAPCs). The fact that we found MAPC-mediated therapeutic effects without detectable presence of MAPCs in the brain after 7 days of onset of hypoxia-ischemia, suggests that the neuroprotective effects of MAPCs last longer than their presence. We postulate that this phenomenon can be explained by MAPCs dampening the detrimental peripheral and cerebral inflammatory responses at an early stage, before these responses can aggravate hypoxic-ischemic injury in the preterm brain.

Our study has several limitations. We did not perform time series of histologic analysis to assess temporal dynamics of global HI-induced injury, the cerebral and peripheral inflammatory response and therapeutic effects of intravenous MAPC administration. Moreover we did not discriminate between the therapeutic effect between the first and second dose of MAPCs. These limitations are inherent to limited experimental groups to

make large animal experiments feasible. Unfortunately, no ovine-specific antibodies are currently available to detect M1 and M2 microglial phenotypes in our studies.

Our findings are clinically highly relevant for several reasons. First, we tested our hypothesis in a well-established pre-clinical animal in which global HI was induced by transient umbilical cord occlusion accurately mimicking the common etiology of hypoxic-ischemic brain injury in preterm infants.^{44, 45} Second, this pre-clinical animal model allows for continuous *in utero* registration of relevant parameters (i.e. vital parameters and aEEG) with strong clinical relevance and predictive value, which gives more insight in HI-induced pathophysiology and efficacy of therapeutic options in a preclinical setting. Third, preclinical studies have confirmed safety and genomic stability of clinical grade MAPCs^{5, 23} and clinical-grade MAPC therapy is currently being tested in phase I/II clinical trials for the treatment of ischemic stroke, graft-versus-host disease (GVHD), acute myocardial infarction and inflammatory bowel disease⁴, emphasizing clinical applicability of these cells.

In conclusion, we have shown in a pre-clinical animal model that MAPCs improve the functional and structural outcome of the preterm brain after global HI. Future studies should further establish the mechanism and long-term therapeutic effects of MAPC-induced neuroprotection in the developing preterm brain exposed to hypoxia-ischemia. Our study may form the basis for future clinical trials, which will evaluate whether MAPC therapy is capable of reducing neurological sequelae in preterm infants with hypoxic-ischemic brain injury.

Conclusions

In conclusion, we have shown in a pre-clinical animal model that MAPCs improve the functional and structural outcome of the preterm brain after global HI. Future studies should further establish the mechanism and long-term therapeutic effects of MAPC-induced neuroprotection in the developing preterm brain exposed to hypoxia-ischemia. Our study may form the basis for future clinical trials, which will evaluate whether MAPC therapy is capable of reducing neurological sequelae in preterm infants with hypoxic-ischemic brain injury.

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3

Mesenchymal Stromal Cell-derived Extracellular Vesicles Protect the Fetal Brain after Hypoxia-Ischemia

Based on:

Mesenchymal Stromal Cell-derived Extracellular Vesicles Protect the Fetal Brain after Hypoxia-Ischemia.

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ABSTRACT

Preterm neonates are susceptible to perinatal hypoxic-ischemic brain injury, for whom currently no treatment is available. In a preclinical animal model of hypoxic-ischemic brain injury in ovine fetuses we have previously demonstrated the neuroprotective potential of systemically administered mesenchymal stromal cells (MSCs). The mechanism of MSC treatment is unclear but suggested to be paracrine through secretion of extracellular vesicles (EVs). We therefore investigated in the current study the protective effects of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in a preclinical model of preterm hypoxic-ischemic brain injury. Ovine fetuses were subjected to global hypoxia-ischemia through transient umbilical cord occlusion, followed by *in utero* intravenous administration of MSC-EVs. The therapeutic effects of MSC-EV administration were assessed by analysis of (electro-) physiological parameters and histology of the brain.

Systemic administration of MSC-EVs improved brain function by reducing the total number and duration of seizures, and by preserving baroreceptor reflex sensitivity. These functional protections were accompanied by a tendency to prevent hypomyelination. Cerebral inflammation remained unaffected by the MSC-EV treatment.

Thus, our data demonstrate that MSC-EV treatment might provide a novel strategy to reduce the neurological sequelae following hypoxic-ischemic injury of the preterm brain. Our study suggests that a cell-free preparation comprising neuroprotective MSC-EVs could substitute MSCs in the treatment of preterm neonates with hypoxic-ischemic brain injury, thereby circumventing the potential risks of systemic administration of living cells.

INTRODUCTION

Hypoxia-ischemia (HI) in the developing brain has been strongly correlated with morbidity and mortality in premature and full-term infants¹. One of the induced pathologies is hypoxic ischemic encephalopathy (HIE), which is associated with adverse neurodevelopmental outcomes, resulting in enormous physical, psychological and economic burden^{1, 2}. Currently, therapeutic intervention strategies for HIE are limited. Term and late preterm neonates suffering from moderate or severe HIE are eligible for 72 h of therapeutic cooling, which has been shown to improve outcomes^{1, 3, 4}. Preterm neonates born before 35 weeks gestational age, however, are still excluded from this therapy⁵. Thus, new treatment strategies are urgently warranted.

In a preclinical animal model of HIE, we have demonstrated that systemic administration of mesenchymal stromal cells (MSCs) promoted functional recovery and prevented structural injury in the preterm brain following global hypoxia-ischemia⁶. These effects were, in part, attributable to reduction of (neuro-) inflammatory processes coordinated by a splenic response⁶. However, the exact mechanisms of action of the exogenously delivered MSCs are not clear.

Initially, MSCs were considered to home into affected tissues and substitute lost cell types. Increasing evidence, however, indicates that MSCs rather exert their therapeutic effects in a paracrine manner via secretion of small extracellular vesicles (EVs) (i.e. exosomes (70-150 nm in diameter) and microvesicles (100-1000 nm))⁷⁻¹⁴, suggesting that administration of intact MSCs is not mandated to exert therapeutic effects. In comparison to MSCs, mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) have been shown to elicit similar biological effects after administration in various preclinical disease models, including models for kidney, cardiac and brain injury^{9, 15-21}. Recently, a therapy-refractory graft-versus-host disease patient was successfully treated with allogeneic MSC-EVs with immune suppressive effects *in vitro* and *in vivo* without showing any side effects²².

Thus, the aim of this study was to assess the therapeutic efficacy of MSC-EVs in preterm brain injury. We hypothesized that systemic administration of MSC-EVs, the paracrine mediators of MSCs, would be neuroprotective in hypoxic-ischemic injury in the preterm brain. We tested this hypothesis in a well-established preclinical animal model, in which hypoxic-ischemic brain injury was induced through transient umbilical cord occlusion (UCO) in the preterm ovine fetus²³. The therapeutic effects of systemic administration of MSC-EVs on brain function were determined by evaluation of seizure burden, a measure for cortical function, and by calculating baroreflex mediated heart rate response (baroreceptor reflex sensitivity), which is needed to regulate blood pressure fluctuations, as a measurement for function of the brainstem. Structural injury was investigated by histological examination of the subcortical white matter. Anti-inflammatory effects were

assessed with histology for microglia in the hippocampus and subcortical white matter, and detection of cerebral infiltration of T lymphocytes.

MATERIALS AND METHODS

Study approval

Experimental procedures and study design were in line with the institutional guidelines for animal experiments, and approved by the Animal Ethics Committee of Maastricht University, The Netherlands.

Randomization and blinding

24 singleton fetuses of time-mated Texel ewes were randomized in four experimental groups: (1) sham umbilical cord occlusion, saline treatment (sham-SAL n=6), (2) sham umbilical cord occlusion, MSC-EV treatment (sham-MSC-EV, n=6), (3) umbilical cord occlusion, saline treatment (HI-SAL, n=6), and umbilical cord occlusion, MSC-EV treatment (HI-MSC-EV, n=6) (figure 1). The investigator performing the (sham) umbilical cord occlusions was blinded to treatment allocation. Tissue sampling and post mortem analyses were conducted in a blinded fashion. A drop-out of 16% (n=4) was observed which was primarily restricted to the sham-MSC-EV group: (i.e. 3 animals of the sham-MSC-EV group and 1 animal of the HI-MSC-EV). Importantly, autopsy of these animals revealed that fetal death was exclusively caused by a single technical reason namely arterial catheter not in situ resulting in exsanguination and subsequent fetal death, thereby excluding any other cause.

Animals and surgery

Singleton fetuses were surgically instrumented at 102 days gestational age (term ~147 days) as described previously²³ (figure 1). In short, fetuses were exposed through a midline laparotomy of the ewe. Umbilical vessel catheters (1.2 mm, Covedien, Mansfield, Massachusetts, USA) were inserted in the femoral artery and brachial vein for blood pressure recordings, blood sampling, and administration of MSC-EVs. Three electrocardiogram (ECG) electrodes were attached to the fetal chest for cardiologic monitoring. Two pairs of custom-made shielded silver-tipped electroencephalogram (EEG) electrodes (Cooner wire Co., Chatsworth, CA, USA) were placed bilaterally on the dura over the parasagittal cortex. An additional reference electrode was placed in the neck²³.

An inflatable vascular occluder (OCD16HD, 16 mm, In Vivo Metric, Healdsburg, Ca. USA) was placed around the umbilical cord for induction of transient global HI.

An additional catheter was placed in the amniotic sac for recordings of amniotic pressure. All leads were exteriorized through a trocar hole in the maternal flank. Post-operatively, the sheep were housed individually with access to food and water *ad libitum*.

Experimental design

After a four day recovery period, the fetuses (106 days GA, experimental day 0) were subjected to 25 minutes of (sham) umbilical cord occlusion by rapid inflation of the vascular occluder with a predefined volume of sterile saline (figure 1). Occlusion was confirmed by an acute drop in heart rate and a gradual decline of blood pressure. Furthermore, global hypoxia-ischemia was monitored with subsequent arterial blood gas analysis (data not shown).

Animals that were randomized to receive MSC-EV treatment received 1 aliquot of 4.0×10^7 cell equivalents divided over two boluses of 2.0×10^7 cell equivalents. The first bolus of 2.0×10^7 cell equivalents was administered 1 hour following (sham) umbilical cord occlusion. The second bolus was administered 4 days after the hypoxic-ischemic insult. Control animals received an equal volume of sterile 0.9% NaCl intravenously at the designated time points. The fetuses were sacrificed 7 days after (sham) UCO and prepared for tissue sampling.

Data acquisition and analysis

Blood pressure, amniotic pressure, ECG and EEG data were acquired and digitized by a custom-made MPAQ unit (Maastricht-Programmable AcQuisition system, Maastricht Instruments BV, Maastricht, The Netherlands) with IDEEQ software (Maastricht Instruments BV) and stored for off-line analysis, as described in detail previously^{6, 23}. Briefly, following detection and removal of high-voltage (EEG signal $>1000 \mu\text{V}$) and flat line artefacts, raw signals were converted into amplitude-integrated EEG (aEEG) traces. The aEEG processing includes an asymmetric band-pass filter that strongly attenuates activity below 2 Hz and above 15 Hz, semi-logarithmic amplitude compression and time compression. As in the neonatal intensive care unit, these aEEG traces were used to detect electrographic seizure activity, which is characterized by an abrupt rise in the lower and upper margin amplitude of the aEEG. Electrographic seizure activity with a duration ≥ 10 s was annotated using aEEG/EEG traces and was performed by a neonatologist, experienced in neonatal aEEG interpretation.

To quantify baroreflex mediated heart rate response (baroreceptor reflex sensitivity, ms/mm Hg), the ratio between the standard deviation of the mean heart rate (R-R intervals) variability and the standard deviation of the mean systolic blood pressure (SD-SDP) was calculated, as reported previously^{24, 25}.

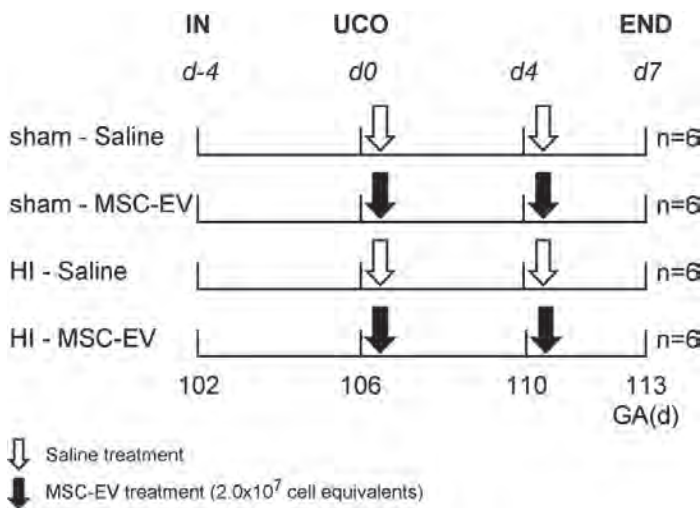


Figure 1. Study design.

Fetuses were instrumented at a gestational age of 102 days. After a recovery period of 4 days fetuses were subjected to 25 min of umbilical cord occlusion or sham occlusion (d0). One hour and 4 days (d4) after umbilical cord occlusion or sham occlusion, fetuses received either intravenous MSC-EVs (2.0×10^7 cell equivalents, closed arrow) or saline 0.9% (open arrow). After a 7 day reperfusion period brain tissue was collected. IN = instrumentation; UCO = umbilical cord occlusion; END = end of experiment; HI = hypoxia-ischemia, SAL = saline, MSC-EV = mesenchymal stem cell-derived extracellular vesicles; GA(d) = gestational age (days).

MSC-EVs

Human bone marrow-derived MSCs were raised from bone marrow donors after informed consent according to the Declaration of Helsinki as described previously²². Briefly, MSCs were expanded in MSC basal media (Pan Biotech, Aidenbach, Germany) supplemented with 10% human thrombocyte lysate, 1% glutamine, and 1% penicillin-streptomycin (Invitrogen, Darmstadt, Germany). Their MSC nature was confirmed by flow cytometry with fluorescent labeled anti-CD44, anti-CD73, anti-CD90, anti-CD105 and anti-CD146 antibodies as positive markers as well as with by anti-CD14, anti-CD31 and anti-CD45 antibodies as negative controls. In addition their osteogenic and adipogenic differentiation potential was confirmed in conventional MSC differentiation assays. MSC-EVs were harvested from MSC-conditioned media which were collected from passage 3 onwards, exactly as described before.² Briefly, media exchanges were performed every 48 hours. MSC-conditioned media were passed through a $0.22 \mu\text{m}$

filter membrane (TPP, Trasadingen, Switzerland) and stored at -20°C until usage. Upon processing all conditioned media samples were thawed and processed at once.

After adding polyethylene glycol (PEG) and NaCl to a final concentration of 10% PEG 6000 (v/v) and 75 mM NaCl over-night incubation at 4°C, MSC-EVs were concentrated by low speed centrifugation (30 min at 1,500 g). Pellets were washed in 0.9% NaCl twice and resuspended in 0.9% NaCl. MSC-EVs were stored as aliquots of 1 mL, each containing the MSC-EV equivalents harvested from the 48 h conditioned media of 4×10^7 cells. Aliquots were stored at -80°C until usage. The obtained MSC-EV fraction was characterized as presented in²² by Nanoparticle Tracking Analyses (NTA)²⁶ and Western Blot (Supplementary figure 1) as described previously.²² Briefly, protein concentrations were determined by the micro BCA assay (Thermo Fisher Scientific, Schwerte, Germany). 5 µg of the concentrated MSC-EVs were treated with sample buffer (DTT, 0.1% SDS, 0.1 M Tris HCl, pH 7.0) and boiled for 5 min at 95°C. Samples were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked in 5% skimmed milk in PBS followed by incubation with antibodies recognizing Tsg101 (Sigma-Aldrich) or CD81 (BD). Subsequently, membranes were incubated with appropriate HRP-conjugated secondary antibodies (Dianova, Hamburg, Germany). Visualization was accomplished by enhanced chemiluminescence (Thermo Fisher Scientific). Furthermore, it was negatively tested for the presence of bacteria, viruses and endotoxins.

Immunohistochemistry in the brain

Right cerebral hemispheres were immersion fixated in 4% paraformaldehyde and subsequently embedded in gelatin. Serial coronal sections (50 µm) were cut on a Leica VT 1200S vibrating microtome (Leica Biosystems, Nussloch, Germany). Free-floating sections at the level of mid-thalamus and posterior hippocampus were stained for ionized calcium binding adaptor molecule (IBA-1) (Wako Pure Chemical Industries, Osaka, Japan) for microglia or for myelin basic protein (MBP) (Merck Millipore, Billerica, MA USA) for white matter, and cleaved caspase-3 for apoptosis (Cell Signaling Technology, Boston, USA) as previously described.^{23, 27}

Paraffin embedded brain coronal sections at the level of mid-thalamus and posterior hippocampus were stained for T cells with CD3 (DAKO A0452, DAKO, Denmark) as reported previously.⁶ Briefly, endogenous peroxidase was inactivated by incubation with 0.3% H₂O₂, dissolved in PBS. Prior to blocking of nonspecific binding with 5% bovine serum albumin (BSA) and incubation with the primary CD3 antibody, antigen retrieval was performed in sodium citrate buffer (pH 6.0) using a microwave oven. Following overnight incubation with the primary antibody, sections were incubated with the appropriate secondary biotin-labeled antibody. Immunostaining was enhanced with

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Vectastain ABC peroxidase elite kit (PK-6200, Vector Laboratories) followed by NiDAB staining and counterstaining with 0.1% nuclear fast red.

Analysis immunohistochemistry

Digital images of the hippocampus (IBA-1, 20 times magnification) and subcortical white matter (SCWM) (IBA-1, MBP, and caspase-3, 100 times magnification) were acquired on an Olympus AX-70 microscope (Olympus, Tokyo, Japan) equipped with a black-white digital camera.

Areal fractions of IBA-1 and MBP immunoreactivity, expressed as the percentage immunoreactivity of the total area using a standard threshold, were determined with Leica Qwin Pro V 3.5.1 software (Leica, Rijswijk, the Netherlands) as described previously.²³

Numbers of Caspase-3 positive cells were determined with Leica Qwin Pro V 3.5.1 software, and averaged to obtain cell numbers for Caspase-3 per section.

For the analysis of CD3 staining in the SCWM, 2 coronal sections per animal were studied at the mid thalamus and posterior hippocampus level. CD3 positive cells were counted at 200 times magnification in ten fields of view on a Leica DM2000 microscope equipped with a digital camera, focused on cerebral vasculature. Averages per animal were expressed as cells per field of view.

Statistics

Animal characteristics and histological outcome parameters are shown as means with 95% Confidence Interval (CI). Comparisons between groups of all parameters were drawn with analysis of variance (ANOVA) or with random intercept models in case of repeated measurements per animal (e.g. different sections per brain) as described previously.²⁸ Data, variables, whose distributions were positively skewed, were log-transformed for statistical testing. For interpretation purposes, averages on the log scale were transformed back to original scale and are presented as geometric means and corresponding 95% confidence intervals.⁶

Seizure data, cumulative throughout the entire experiment, showed right-skewness due to the absence of seizures in non-HI groups that could not be corrected by log-transformation. Therefore, group's comparisons were performed with Mann-Whitney tests. Seizure burden is represented as median and interquartile range (IQR). $P < 0.05$ was considered statistically significant. All statistical analyses have been performed with IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA).

Statistical analysis of baroreceptor reflex sensitivity (BRS) was performed using a Bayesian multilevel model.²⁹ Statistical time series analysis was conducted using the Stan for R package (version 2.6.0) in R (version 3.1.1).

RESULTS

Animal characteristics

To assess the neuroprotective capacities of MSC-EVs, we randomized 24 preterm ovine fetuses in different experimental groups (figure 1). Fetuses were subjected to 25 minutes of global hypoxia-ischemia (HI) through umbilical cord occlusion (UCO) or sham UCO. MSC-EVs were administered 1 hour and four days after UCO (figure 1).

Brain to bodyweight ratios did not differ significantly between the experimental groups. Splenic weight relative to bodyweight, indicative of activation of the splenic inflammatory response²³ was significantly reduced after global HI (sham-SAL vs. HI-SAL; $P<0.05$), indicative of splenic involution, which is an indication of activation of the peripheral immune system.^{23, 30} MSC-EVs prevented HI-induced splenic involution (HI-SAL vs. HI-MSC-EV; $P<0.05$) (table 1).

Table 1. Animal characteristics.

	sham - SAL	sham – MSC-EV	HI - SAL	HI - MSC-EV
Brain/Bodyweight ratio (g/kg BW)	16.77 (14.80 ; 18.74)	21.19 (10.48 ; 31.90)	16.19 (13.50 ; 18.88)	16.70 (11.27 ; 22.13)
Spleen/Bodyweight ratio (g/kg BW)	2.68 (1.82 ; 3.54)	3.10 (0.25 ; 5.95)	1.72 (1.45 ; 1.99)*	2.43 (2.03 ; 2.83)[#]

Brain weight corrected for bodyweight was unaffected by global HI and/or MSC-EV treatment. Splenic weight corrected for bodyweight significantly reduced following global HI, which was significantly restored after MSC-EV treatment. Ratios are depicted as means with 95% CI. * $P\leq 0.05$ compared to control (sham-SAL), [#] $P\leq 0.05$ compared to HI-SAL). BW = bodyweight; HI = hypoxia-ischemia; SAL = saline; MSC-EV = mesenchymal stem cell-derived extracellular vesicles.

MSC-EVs prevent loss of cortical function and baroreflex sensitivity after global HI

Electrographic seizure burden was assessed as a measure of cortical function, and was investigated by amplitude integrated electroencephalogram (aEEG) for total numbers of seizures (figure 2A) and total amount of time of seizure activity (figure 2B). Global HI induced a profound increase in the cumulative number of seizures (sham-SAL vs. HI-SAL; $P=0.003$) and the cumulative duration (sec.) of seizure activity (sham-SAL vs. HI-SAL; $P=0.002$) which were reduced by MSC-EV treatment, (cumulative number: HI-SAL vs. HI-

MSC-EV; $P=0.021$) (cumulative duration: HI-SAL vs. HI-MSC-EV; $P=0.029$). No differences in seizure burden between the sham conditions could be detected.

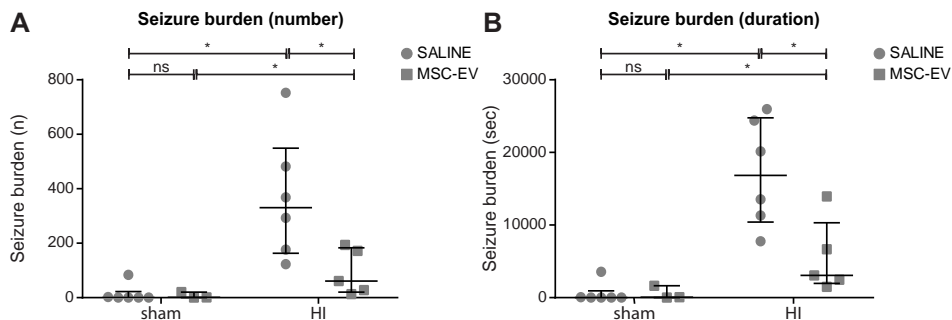


Figure 2. MSC-EV treatment induces functional neuroprotection after global HI.

Global HI caused a significant seizure burden indicated by an increased (A) total number and (B) duration of seizures compared to sham occluded animals. Administration of MSC-EVs reduced electrographic seizure number and duration compared to saline treated animals. Medians \pm interquartile ranges and levels of significance, which were calculated by Mann-Whitney test, are depicted. * $P \leq 0.05$; NS = not significant. HI = hypoxia-ischemia; MSC-EV = mesenchymal stem cell-derived extracellular vesicles.

Brain stem function was assessed by analysis of baroreceptor reflex sensitivity (figure 3). The baroreflex is a vital part of the vascular auto regulatory system, ensuring adequate perfusion of the fetal brain upon disturbance of homeostasis.²⁴ Baroreceptor reflex sensitivity was reduced at experimental day 4 days following global HI when compared to controls and remained compromised throughout the experiment. MSC-EV treatment prevented HI-induced compromise of the baroreceptor reflex sensitivity from experimental day 3 onwards. Surprisingly, MSC-EV treatment significantly reduced baroreceptor reflex sensitivity in healthy controls.

MSC-EVs partially protect against HI-induced hypomyelination, but not against apoptosis

Myelin basic protein (MBP) immunoreactivity, which was analyzed to determine white matter injury, was assessed in the subcortical white matter (SCWM) (figure 4 A-B). Global HI resulted in marked hypomyelination in the SCWM as indicated by a significant decrease of MBP immunoreactivity (sham-SAL vs. HI-SAL; $P=0.001$). MSC-EV treatment showed a mild trend to protect against hypomyelination, however statistical significance was not reached (HI-SAL vs. HI-MSC-EV; $P=0.100$).

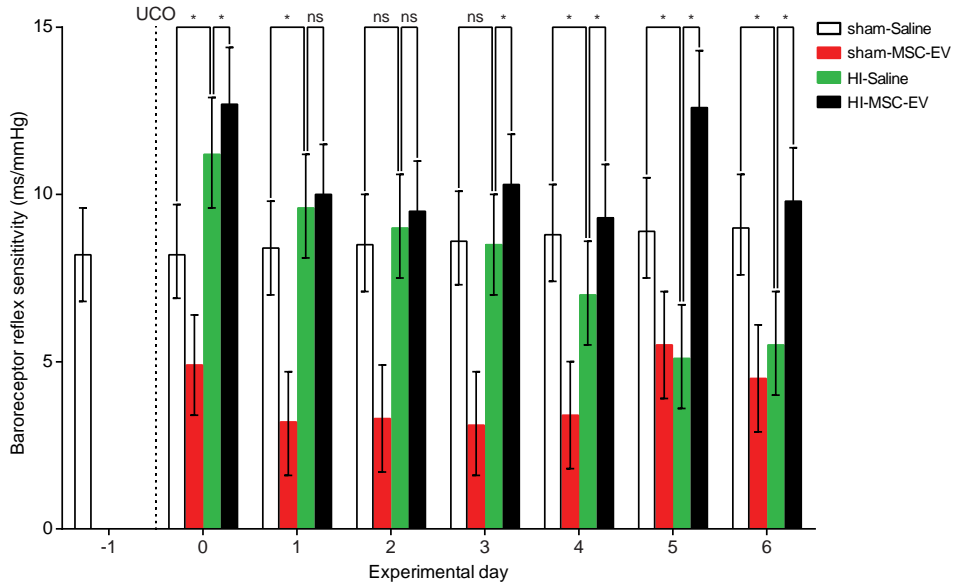


Figure 3. MSC-EVs prevented loss of baroreceptor reflex sensitivity.

Global HI (green bars) caused a significant gradual decline of baroreceptor reflex sensitivity over time, which was prevented by MSC-EV treatment (black bars); means \pm 95% CI and levels of significance of the treatment effect (HI-SAL vs. HI-MSC-EV) are depicted, which were calculated by the Bayesian multi-level model. MSC-EV treatment significantly compromised baroreceptor reflex sensitivity in healthy controls (red bars). * $P < 0.05$; NS = not significant. UCO = umbilical cord occlusion; HI = hypoxia-ischemia; MSC-EV = mesenchymal stromal cell-derived extracellular vesicles.

Assessment of cleaved caspase-3 in the SCWM was performed as a marker for apoptotic cell death. The numbers of caspase-3-positive cells following global HI tended to be higher at 7 days in comparison to controls ($P = 0.070$). There was no effect on the numbers of caspase-3-positive cells after hypoxia-ischemia by MSC-EV treatment (HI-SAL vs. HI-MSC-EV; $P = 0.457$).

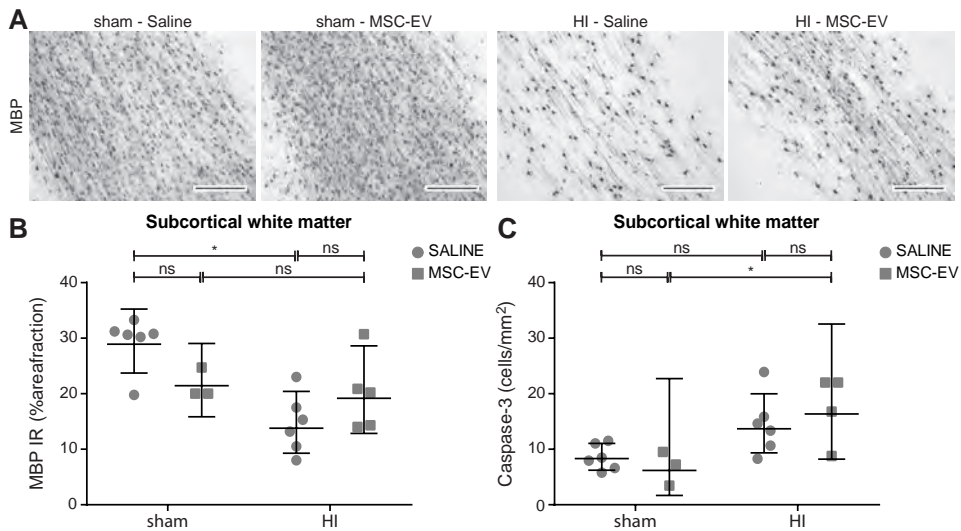


Figure 4. MSC-EVs reduced white matter injury after global HI, but did not prevent HI-induced apoptosis. (A) Immunohistochemical MBP staining in the subcortical white matter of all four experimental groups at 100x magnification. Global HI induced marked hypomyelination in the subcortical white matter. MSC-EVs tended to prevent the decrease in MBP reactivity after global HI. The area fraction of MBP was similar in sham conditions. (B) Graphical representation of MBP immunoreactivity in the subcortical white matter. (C) Graphical presentation of caspase-3 cell-density in the subcortical white matter. A mild trend in increase of caspase-3 cell density was found following global HI. MSC-EV treatment did not affect apoptosis in the subcortical white matter. Levels of significance are depicted, which were calculated by the random intercept model with all repeated measures (i.e. brain sections) per animal (sham-SAL=6, sham-MSC-EV=3, HI-SAL=6, HI-MSC-EV=5). Due to positive skewing, data was log-transformed for statistical testing. For graphical presentation and interpretation, averages on the log scale were transformed to the original scale and presented as geometric means with corresponding 95% confidence intervals. * $P \leq 0.05$; NS = not significant. MBP = myelin basic protein 1; HI = hypoxia-ischemia; MSC-EV = mesenchymal stem cell-derived extracellular vesicles; IR = immunoreactivity. Scale bar = 200 μ m.

MSC-EVs do not protect against HI-induced neuroinflammation

We assessed neuroinflammation in the SCWM (figure 5A-C) and the hippocampus (figure 5B-D) by analyzing immunoreactivity of ionized calcium binding adaptor protein 1 (IBA-1). Global HI induced a marked increase of IBA-1 immunoreactivity in the SCWM (sham-SAL vs. HI-SAL; $p=0.001$) and the hippocampus (sham-SAL vs. HI-SAL; $p<0.0001$), indicative of profound microglial activation and proliferation. MSC-EV treatment had no effects on hippocampal IBA-1 immunoreactivity (HI-SAL vs. HI-MSC-EV; $P=0.398$), but increased IBA-1 immunoreactivity in the SCWM (HI-SAL vs. HI-MSC-EV; $P=0.041$). MSC-EV treatment had no effects on IBA-1 immunoreactivity in both the SCWM and hippocampus in sham occluded animals compared to controls.

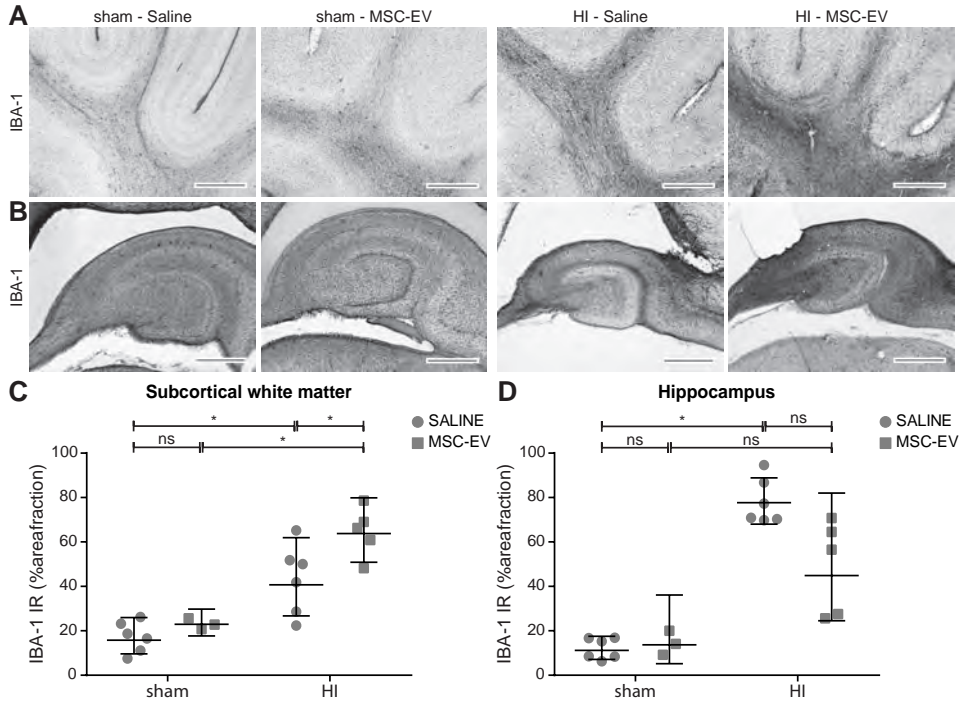


Figure 5. MSC-EVs do not reduce cerebral inflammation in the subcortical white matter and hippocampus. (A-B) Immunohistochemical of IBA-1 staining. Global HI induced a significant increase of IBA-1 immunoreactivity in (A) the subcortical white matter (100x magnification) and (B) the hippocampus (20x magnification) which was not attenuated by MSC-EV treatment. (C-D) Graphical presentation of area fraction of IBA-1 immunoreactivity in (C) the subcortical white matter and (D) the hippocampus. Levels of significance are depicted, which were calculated by the random intercept model with all repeated measures (i.e. brain sections) per animal (sham-SAL=5, sham-MSC-EV=3, HI-SAL=6, HI-MSC-EV=5). Due to positive skewing, data was log-transformed for statistical testing. For graphical presentation and interpretation, averages on the log scale were transformed to the original scale and presented as geometric means with corresponding 95% confidence intervals. * $P \leq 0.05$; NS = not significant. IBA-1 = ionized calcium binding adaptor molecule 1; HI = hypoxia-ischemia; MSC-EV = mesenchymal stem cell-derived extracellular vesicles, IR = immunoreactivity. Scale bar = 1000 μ m.

Finally, we assessed the influx of T lymphocytes in the fetal brain by immunohistochemical analysis for CD3 in the SCWM (figure 6). Cerebral influx of CD3 positive cells tended to increase following global HI (sham-SAL vs. HI-SAL; $p=0.078$). Remarkably, MSC-EV treatment resulted in an increase of CD3 positive cells in the SCWM of sham occluded animals (sham-SAL vs. sham-MSC-EV; $P=0.013$).

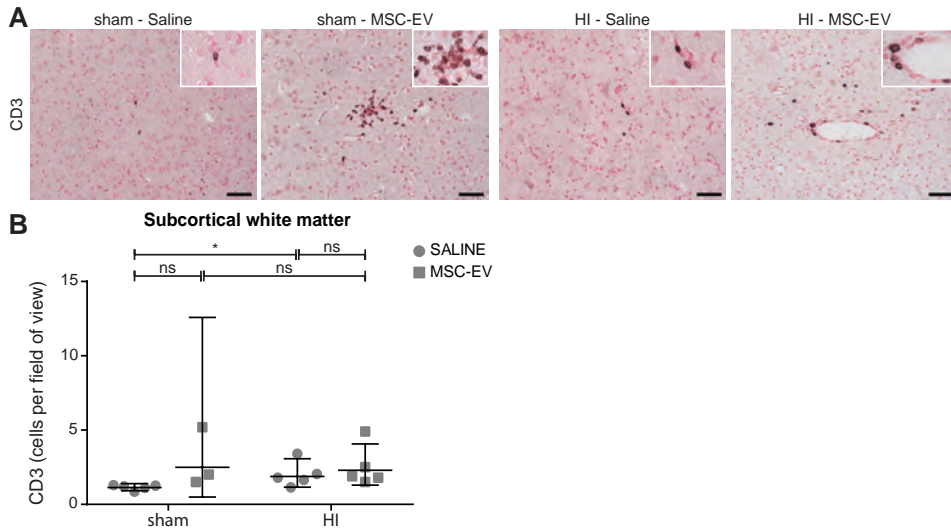


Figure 6. MSC-EVs increase cerebral influx of T-effector cells.

(A) Immunohistochemical staining for CD3 positive T lymphocytes in the subcortical white matter of all four experimental groups at 200x magnification (insert 400x magnification). (B) Graphical representation of CD3 positive cells per field of view in the subcortical white matter. MSC-EV treatment increased numbers of CD3 positive T-lymphocytes in the subcortical white matter compared to saline treated animals. Levels of significance are depicted, which were calculated by the random intercept model with all repeated measures (i.e. brain sections) per animal (sham-SAL=5, sham-MSC-EV=3, HI-SAL=5, HI-MSC-EV=5). Due to positive skewing, data was log-transformed for statistical testing. For graphical presentation and interpretation, averages on the log scale were transformed to the original scale and presented as geometric means with corresponding 95% confidence intervals. * $P \leq 0.05$; NS = not significant. HI = hypoxia-ischemia; MSC-EV = mesenchymal stem cell-derived extracellular vesicles. Scale bar = 100 μ m.

DISCUSSION

In the current study we showed that intravenous administration of MSC-EVs prevented functional impairment and showed a tendency to protect against structural injury of the preterm brain after global HI apparently without reducing cerebral inflammation. These findings are clinically highly relevant since post-ischemic seizure activity is associated with adverse neurodevelopmental outcomes.³¹⁻³⁴ The baroreceptor reflex buffers short-term changes in blood pressure by adapting systemic vascular resistance, myocardial contractility and heart rate. The clinical relevance of an impaired baroreceptor reflex function relates to the increased risk for developing additional brain injury by exposing the vulnerable developing cerebral vascular network to (large) fluctuations in blood pressure. In preterm infants, fluctuations associated with high blood pressure may disrupt the cerebral capillaries in the germinal matrix while periods of low blood pressure may cause localized hypoxia-ischemia of the watershed areas.³⁵ Moreover, preterm infants display fluctuating pressure-passivity between systemic blood pressure

and cerebral blood flow representing a considerably increased risk of cerebral hemorrhage or hypoxia.³⁶ Previously, we have shown that HI reduces the baroreceptor reflex mediated heart rate response.²⁴ In this paper, we demonstrate for the first time that MSC-EV treatment results in preservation of baroreceptor reflex sensitivity after exposure to global HI. Our data suggest a wide-spread functional protection by MSC-EVs of the central nervous system after global HI. Remarkably, we found that MSC-EV treatment reduced baroreceptor reflex sensitivity in healthy controls suggesting that MSC-EV treatment should be given with care in order to avoid adverse effects, and warranting further analyses of the effects of MSC-EVs on the developing brain in preclinical animal models before introducing MSC-EVs into clinical trials. However, clinical data in an adult GVHD patient demonstrated safe intravenous administration of MSC-EVs without adverse cardiovascular and hemodynamic outcomes.²²

Consistent with improved electro cortical function and preservation of baroreceptor reflex sensitivity, we demonstrated that intravenous administration of MSC-EVs tended to protect against HI-induced white matter injury, which is the clinical hallmark of neonatal HI brain injury.³⁷ This study suggests that the functional neuroprotective effects of MSC-EVs are not primarily caused by anti-inflammatory mechanisms. This is in contrast to our previous results in which the neuroprotective effects of intravenously administered MSCs could be, at least in part, attributed to anti-inflammatory capacities of MSCs⁶. Although prevention of splenic involution⁶, might indicate inhibition of systemic immune activation³⁰, this effect was not paralleled by anti-inflammatory modifications of the ischemic fetal brain. This latter finding is in contrast with results from our previous study where splenic involution was associated with prevention of cerebral inflammation after systemic MSC administration. This difference in anti-inflammatory properties of MSCs-EVs in the current and MSC in the previous study could be explained by two reasons: First, the immune modulatory properties of MSCs and their EVs differ between independent MSC preparations.²² Second, in contrast to MSC-EVs, MSCs can sense micro environmental conditions to which they are exposed (e.g. licensing); a pro-inflammatory environment MSCs stimulates polarization towards an anti-inflammatory phenotype, whereas alternative stimulation has been reported to rather propagate a pro-inflammatory phenotype.³⁸⁻⁴⁵ This may affect their secretion of therapeutically active immune modulatory components. However, recently we compared the therapeutic effect of MSC-EVs and that of corresponding MSCs in a murine ischemic stroke model and did not recognize any differences.²¹ Although, such a side by side comparison was not performed in our model, this latter study suggests that differences between the two studies cannot be attributed to licensing.

The concept of inflammation-independent effects as neuroprotective mechanisms of MSC-EVs therapy is supported by several studies showing that MSC-EVs can prevent apoptosis and stimulate angiogenesis after hypoxia-ischemia.^{16, 20, 46-50} However, no effects on angiogenic markers (mRNA levels of vascular endothelial growth factor

(VEGF) A and VEGF-receptor 2; data not shown) or on the number of apoptotic cells after global HI with MSC-EV treatment were found in our study, suggesting that the neuroprotective potential of MSC-EV cannot be attributed to anti-apoptotic or angiogenic properties.

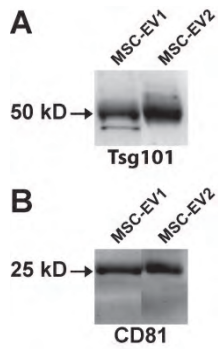
Despite the identified discrepancies in therapeutic effects between MSCs and MSC-EVs used in the current study, the application of EVs offers several advantages over the administration of MSCs: (1) the risk of malignant transformation is greatly reduced because EVs are non-self-replicating.⁵¹ (2) Lacking an own metabolism, the EVs activity can hardly be influenced by the *in vivo* environment in patients, thus allowing for a much better characterization of their functional properties. (3) Owing to their small size EVs are less likely to generate emboli upon intravenous administration, as may be the case with MSCs. (4) In addition, EVs can be sterilized by filtration. Thus, from a regulatory point of view, the production and especially the quality control of EV fractions for clinical treatment application is less complicated than for a cellular therapeutic of *in vitro* expanded cells.⁵² (5) Last but not least, EVs can be developed independently of the original donor as they are derived from MSCs from unrelated donors and thus offer the opportunity to be turned into an “off-the-shelf” product.

We have chosen this well-established preclinical sheep model since it enables us to accurately mimic the etiology of hypoxic-ischemic injury of the developing preterm brain, including continuous registration of clinical parameters with strong clinical relevance and predictive values.^{6, 53, 54} Nevertheless, our study has several shortcomings. We have no information on the dynamics of the effects of MSC-EVs nor have we studied different doses. Time course information is thus desirable in our model. In future experiments, back-to-back comparison of MSCs and their corresponding EV fraction will provide crucial mechanistic insights into the mode of action of MSCs and their EVs. The scope of the current study was however assessing the feasibility of MSC-EV treatment to improve brain function after hypoxia-ischemia.

In conclusion, we have demonstrated in a preclinical animal model that MSC-EVs harbor neuroprotective potential as shown by improved functional and structural outcomes of the preterm brain following global HI which is in line with previous reports.^{16, 17, 19, 22, 55,}

⁵⁶ Future studies to determine the optimal EV-contents and dosing strategy should be performed in order to establish a clinically safe, cell-free therapy for preterm babies after hypoxia-ischemia.

SUPPLEMENTARY FIGURES



Supplementary figure 1. Characterization of MSC-EV fractions with Western blot.

MSC-EV1 was the MSC-EV fraction used in the successful treatment of a GVHD patient²² and was used as a positive control for the MSC-EV fraction used in the current study (labeled as MSC-EV2 in this figure). Obtained MSC-EV fractions were positive for exosomal markers Tsg101 (A) and CD81 (B).

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Chapter 3

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4

Neuroinflammation and Structural Injury of the Fetal Ovine Brain Following Intra-amniotic *Candida albicans* Exposure

Based on:

Neuroinflammation and Structural Injury of the Fetal Ovine Brain Following Intra-amniotic *Candida albicans* Exposure.

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ABSTRACT

Intra-amniotic *Candida albicans* infection is associated with preterm birth and high morbidity and mortality rates. Survivors are prone to adverse neurodevelopmental outcomes. The mechanisms leading to these adverse neonatal brain outcomes remain largely unknown. To better understand the mechanisms underlying *Candida albicans*-induced fetal brain injury, we studied immunological responses and structural changes of the fetal brain in a well-established translational ovine model of intra-amniotic *Candida albicans* infection. In addition, we tested whether these potential adverse outcomes of the fetal brain were improved *in utero* by antifungal treatment with Fluconazole.

Pregnant ewes received an intra-amniotic injection of 10^7 colony forming units *Candida albicans* or saline (controls) at 3 or 5 days before preterm delivery at 0.8 of gestation (term~147 days). Fetal intra-amniotic/intra-peritoneal injections of Fluconazole or saline (controls) were administered 2 days after *Candida albicans* exposure. Post mortem analyses for fungal burden, peripheral immune activation, neuroinflammation and white matter/ neuronal injury were performed to determine the effects of intra-amniotic *Candida albicans* and Fluconazole treatment.

Intra-amniotic exposure to *Candida albicans* caused a severe systemic inflammatory response, illustrated by a robust increase of plasma IL-6 concentrations. Cerebrospinal fluid cultures were positive for *Candida albicans* in the majority of the 3 day *Candida albicans*-exposed animals whereas no positive cultures were present in the 5 day *Candida albicans*-exposed and Fluconazole treated animals. Although *Candida albicans* was not detected in the brain parenchyma, a neuroinflammatory response in the hippocampus and white matter was seen which was characterized by increased microglial and astrocyte activation. These neuroinflammatory changes were accompanied by structural white matter injury. Intra-amniotic Fluconazole reduced fetal mortality, but did not attenuate neuroinflammation and white matter injury.

Intra-amniotic *Candida albicans* exposure provoked acute systemic and neuro-inflammatory responses with concomitant white matter injury. Fluconazole treatment prevented systemic inflammation without attenuating cerebral inflammation and injury.

INTRODUCTION

Preterm birth is associated with chorioamnionitis which is defined as inflammation of the fetal membranes and amniotic fluid caused by microbial invasion.^{1, 2} The microorganisms most frequently associated with this condition include *Ureaplasma species*, *Mycoplasma hominis*, and *Gardnerella vaginalis* all of which most commonly originate from the lower reproductive tract.³ These microorganisms and/or inflammatory mediators in the amniotic cavity can cause a fetal inflammatory response syndrome (FIRS).⁴⁻⁶ Chorioamnionitis and subsequent FIRS are independent risk factors for adverse outcomes, including injury of the fetal brain.^{5, 7} Adverse neurodevelopmental outcomes result from diffuse cerebral inflammation and white matter injury, periventricular leukomalacia and intraventricular hemorrhage.^{5, 8, 9} These conditions are associated with a high mortality rate and survivors are predisposed to long-term morbidity including mental retardation, impaired learning and visual disorders and in severe cases cerebral palsy.^{7, 8, 10}

The pathophysiology of chorioamnionitis can also include viral and fungal species.^{4, 11, 12} *Candida albicans* (*C. albicans*) is a commensal fungus of the gastro-intestinal tract which can be asymptomatic in the vaginal microbiota with increasing incidence during pregnancy.^{13, 14} Intra-amniotic *C. albicans* infections are associated with high mortality rates and severely impaired neurodevelopmental outcomes¹³⁻¹⁶ in which the mechanisms linking fetal exposure to neurological pathologies remain essentially unstudied. We hypothesized that antenatal exposure to *C. albicans* caused a neuroinflammatory response and subsequent white matter injury, which we tested by exposing fetal sheep to intra-amniotic *C. albicans*.^{16, 17}

In two clinical cases intra-amniotic *C. albicans* infections resolved after oral and intra-amniotic Fluconazole.¹³ Fluconazole is the most frequently used antifungal in *C. albicans* infections, and inhibits ergosterol synthesis, which is an essential component of fungal cell membranes.¹⁸ We therefore further hypothesized that fetal intra-amniotic and intra-peritoneal administration of Fluconazole would reduce the neuroinflammatory response and subsequent white matter injury to the fetal brain. Accordingly, systemic immune activation, neuroinflammation and structural white matter injury were assessed in the fetal sheep exposed to intra-amniotic *C. albicans* and treated with Fluconazole.

METHODS

Experimental design

The study was approved by and performed according to the guidelines of the animal ethics committee of the University of Western Australia (Perth, Australia). Twenty-six date-mated Merino ewes with singleton pregnancies were randomly assigned to receive an intra-amniotic injection of saline (2 mL) as a control or *C. albicans* (10^7 colony forming units (CFU), Western Australian clinical isolate).¹⁶ After 2 days an intra-amniotic/-peritoneal injection of Fluconazole (F) (30 mg per injection, Claris Life Sciences Limited, Chacharwadi-vasana, Ahmedabad-382 213, India) or saline (controls) was administered with delivery after 1 and 3 days (Figure 1). Intra-amniotic injections were performed as previously described.¹⁷

We did not include a 5D *C. albicans* only group since previous results with this model indicated that 5 days exposure to *C. albicans* alone was lethal.^{16, 17} Given the survival of 5D *C. albicans*/Fluconazole-treated group, we have concluded that Fluconazole in this model increases survival.^{16, 17}

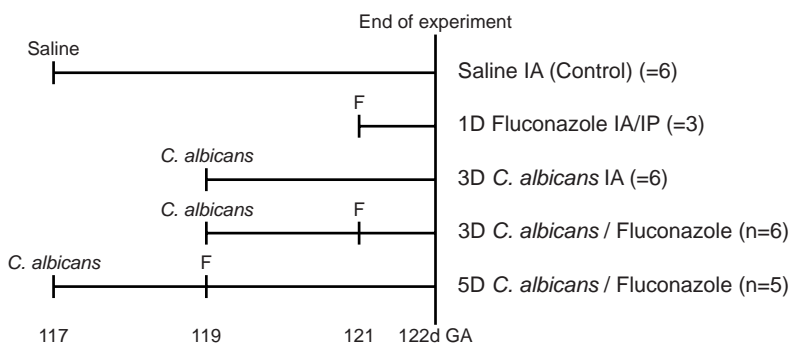


Figure 1 Experimental setup.

Animals were exposed to *C. albicans* for 3 or 5 days in the absence or presence of intra-amniotic (IA) / intraperitoneal (IP) Fluconazole (F). Control animals received intra-amniotic (IA) injection with saline. Fetuses were delivered at 0.8 of gestation.

Tissue collection

Fetuses were delivered by Caesarean section at 0.8 of gestation (term ~147 days)¹⁹, equivalent of 32 - 34 weeks human gestation²⁰, and euthanized with intravenous pentobarbitone (100 mg/kg). Amniotic fluid, blood and cerebrospinal fluid (CSF) were collected at delivery and cultures for *C. albicans* were performed.¹⁶ Briefly, amniotic fluid (100 μ L) was inoculated onto Difco Sabaraud-Dextrose agar and incubated at 37 °C for 48h. Blood (2 mL) and liquor (1 mL) was inoculated into BACTEC Peds Plus culture

vials (Becton Dickinson, Franklin Lakes, NJ) and incubated aerobically at 37 °C for 72 hours.¹⁶ Brains were immersion fixed in 4% paraformaldehyde.

Enzyme-linked immunosorbent assay IL-6.

Plasma interleukin (IL)-6 concentrations were assessed as an indication for systemic inflammation using a sheep-specific sandwich enzyme-linked immunosorbent assay (ELISA). First, 96 well plates were pre-coated with 100 µL of mouse-anti-ovine monoclonal antibody (MAB1004, Millipore, Darmstadt, Germany, working concentration 1:200) overnight at 4°C. Plates were washed three times with 0.05% tween-20 in phosphate buffered saline (PBST). Non-specific binding was blocked with a blocking buffer (1% bovine serum albumin (BSA) in PBST) for 1 hour followed by a washing step.

Protein standards were prepared by serial dilution of recombinant IL-6 (ImmunoChemistry Technologies, Bloomington, MN, USA). Plasma samples (100 µL) were loaded per well in duplicate and incubated for 2 hours at room temperature. Unbound protein was omitted by washing with PBST, followed by incubation with detection antibody (rabbit-anti-ovine IL-6, AB1839, Millipore, Darmstadt, Germany, working concentration 1:500) for 60 minutes and subsequent washing. Next, each well was incubated for 30 minutes with 100 µL of a goat-anti-rabbit-HRP (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA, working concentration 1:500) followed by washing and incubation with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution for 15 minutes at room temperature. The reaction was stopped by addition of 50 µL 2N sulfuric acid to each well. Plates were then read on an ELISA plate reader at 450 nm.

Histology and immunohistochemistry.

The right hemisphere was divided into 4 defined anatomical regions. Serial coronal sections of identical thickness (4 µm) of the region containing the posterior hippocampus and mid-thalamus were cut with a Leica RM2235 microtome. Within this region, we analyzed hippocampus and the cerebral white matter since these regions are most affected following intra-uterine infection at this developmental stage.²¹ All stainings were performed in series (every 15th section) and four sections per staining per animal were analyzed. Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stainings were performed for morphological and anatomical analysis and for identification of pseudohyphae of *C. albicans*, respectively. Adjacent sections were stained for ionized calcium binding adaptor molecule 1 (IBA-1) (Wako Pure Chemical Industries, Osaka, Japan) for microglia, glial fibrillary acidic protein (GFAP) (DAKO Z0334) for astrocytes, myelin basic protein (MBP) (Merck Millipore, MAB386) for myelin

sheaths, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) (Sigma, C5922) for mature myelin producing oligodendrocytes, oligodendrocyte transcription factor 2 (Olig2) (Millipore, AB9610) for oligodendrocyte lineage cells, cluster of differentiation (CD) 68 (DAKO, M0718) for active macrophages, myeloperoxidase (MPO) (DAKO, A0398) for neutrophils, CD3 (DAKO A0452) for T-lymphocytes, Ki67 (DAKO, M7240) for cell proliferation, and cleaved caspase-3 (Cell signalling, #9661) for apoptosis.

Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was inactivated with 0.3% H₂O₂ treatment. For every immunolabeled stain with the exception of GFAP and CD68, antigen retrieval was performed by microwave boiling of tissue sections in citrate buffer, pH 6.0. For CD68, antigen retrieval was performed with proteinase K (DAKO, S3004) treatment for 5 minutes at 37°C. Nonspecific binding was blocked by incubation with 5% BSA (MBP, Olig2 and CD3) or goat serum in PBS (IBA-1, GFAP, CNPase, CD68, MPO and Ki67). Sections were incubated overnight at 4°C in a closed humidity chamber with primary antibody (IBA-1, GFAP, MBP, CNPase and caspase-3 1:1000; Olig2, MPO and CD3 1:200; Ki67 1:100; CD68 1:50), and subsequently incubated with the specific secondary antibody. Immunostaining was enhanced with a Vectastain ABC peroxidase Elite kit (Vector Laboratories Inc, Burlingame, CA) followed by a (nickel) 3,3'-diaminobenzidine (DAB) staining and 0.1% Nuclear Fast Red (Olig2, CD3, Ki67 and caspase-3) or Mayer's Hematoxylin (CD68 and MPO) for background staining. Sections were dehydrated and coverslipped.

To identify the mature and total number of oligodendrocytes, an immunohistochemical procedure for sequential double staining of CNPase and Olig2 was applied. In this procedure, sections were treated as described above and incubated with the anti-CNPase antibody, followed by incubation with a biotinylated anti-mouse secondary antibody and visualization with 3,3-diaminobenzidine (DAB; Dako). Before incubation with the Olig2 primary antibody, slides were blocked again with 5% BSA for 60 minutes. Cells that stained positive for Olig2 were visualised by HistoGreen (Linaris, E109).

Immunofluorescence

To identify *C. albicans* in the brain tissue, immunofluorescence staining was performed as described previously using a rabbit anti-*C. albicans* antibody (Meridian Life Science, Memphis, TN, working concentration 1:50) and appropriate Alexa Fluor 594-labelled secondary antibody (working concentration 1:200).¹⁷ Briefly, sections were deparaffinized, rehydrated and incubated in 0.25% ammonia dissolved in 70% ethanol for one hour on a shaker. Antigen retrieval was performed using citrate buffer (pH 6.0) in a microwave oven. Nonspecific binding was blocked by incubation with 10% goat serum for one hour followed by overnight incubation with primary anti-*C.albicans* antibody dissolved in 0.1% PBS with 0.2% Tween-20. For fluorescence staining, Alexa Fluor conjugated secondary antibody was used followed by incubation in 0.3% Sudan

Black Solution to reduce auto-fluorescence. Counterstaining was done with the fluorescent nuclear marker Hoechst.

To assess neuronal injury, a Fluoro-Jade C staining was performed, which is a specific marker for identification of degenerating neurons.^{22, 23} In this procedure, sections were deparaffinized, rehydrated and immersed in 0.06% potassium permanganate with 0.01% Hoechst for 10 minutes. Before immersing in the Fluoro-Jade C solution, sections were rinsed in running tap water for 1-2 minutes. Immersion in the Fluoro-Jade C solution (Millipore, AG325) (0.01% stock solution in 0.1% acetic acid) was performed in the dark for 10 minutes. Sections were washed with distilled water and subsequently air dried at 50°C. Finally, sections were cleared with xylene for 1 minute before coverslipped using DPX.

Qualitative analysis

H&E-stained sections were analysed by three independent investigators and two neuropathologists, blinded to the experimental groups, to assess overall brain structure and inflammatory changes. The absence of tissue autolysis in our sections was confirmed by neuropathologists. The sections were examined for the presence of hemorrhages, gliosis, (cytotoxic) oedema, abscess formation, and structural damage, including cyst formation. *C. albicans* fluorescent and PAS-stained sections were examined for the presence of *C. albicans*.

Quantitative analysis

For the analysis of IBA-1, GFAP, MBP, CNPase, Olig2, and CD68 immunoreactivity, digital images of the hippocampus, white matter (WM) and periventricular white matter (PVWM) were acquired using an Olympus AX-70 microscope (Olympus, Tokyo, Japan) equipped with a black and white digital camera. From each section, one picture of the hippocampus was taken at a 20x magnification and images in the WM were taken at a 100x magnification of the gliotic foci and 4-6 consecutive images of the PVWM. The area fraction of IBA-1, GFAP and MBP immuno-reactivity was determined with a standard intensity threshold to determine positive staining using Qwin Pro V 3.5.1 software (Leica, Rijswijk, the Netherlands). Blood vessels and artefacts were excluded from analysis. The CNPase and Olig2 positive cells were counted using Qwin software and expressed as cells/mm². Ki67, caspase-3, CD3 and MPO positive cells were counted in all brain regions, focussing on the cerebral vasculature. The digital images were acquired and analyzed by an independent observer who was blinded to the experimental groups. Fluoro-Jade C/Hoechst stainings were examined using the Olympus AX-70 fluorescent microscope.

Data analysis

Statistical analysis was performed with GraphPad Prism software (version v5.0; GraphPad Software Inc., La Jolla, CA, USA). Results were analyzed using the non-parametric Kruskal-Wallis test with Dunnett's post-hoc testing to compare different groups. Results are given as mean and standard error of the mean (SEM) with significance at $p < 0.05$.

RESULTS

C. albicans culture

C. albicans colonial morphology was confirmed by growth on Brilliance Candida Agar (Oxoid, Adelaide, Australia). Positive cerebrospinal fluid (CSF) cultures for *C. albicans* were detected in 67% (4/6) of the 3D *C. albicans*-exposed animals and in 50% (3/6) of the 3D *C. albicans*/Fluconazole-treated animals (Table 1). No positive cultures were present in the 5D *C. albicans*/Fluconazole-treated group (0/5). Control animals had negative CSF cultures. Results of amniotic fluid and blood cultures were previously published by Maneenil and colleagues and are also summarized in the table.¹⁶

Table 1 Candida albicans cultures.

	Control	3D <i>C. albicans</i>	3D <i>C. albicans</i> and Fluconazole	5D <i>C. albicans</i> and Fluconazole
Cerebrospinal fluid	0/6	4/6	3/6	0/5
Fetal blood *	0/6	4/6	5/6	3/5
Amniotic fluid *	0/6	6/6	6/6	5/5

The ratio of *C. albicans* positive / negative cultures are depicted. * Based on previously published data.¹⁶

No evidence of C. albicans invasion in the brain

Despite substantial numbers of animals with positive CSF cultures for *C. albicans*, PAS and immunofluorescent staining did not identify *C. albicans* in the parenchyma of the brains of any of the experimental groups (data not shown).

Pro-inflammatory cytokine response

Significantly elevated plasma IL-6 concentrations were found in 50% (3/6) of the 3D *C. albicans*-exposed animals when compared to controls (Figure 2). 40% (2/5) of the 5D *C. albicans*/Fluconazole-treated animals demonstrated elevated plasma concentrations of IL-6, however statistical significance was not reached. Plasma IL-6 concentrations in the

controls, 1D Fluconazole- and 3D *C. albicans*/Fluconazole-treated animals were not detectable.

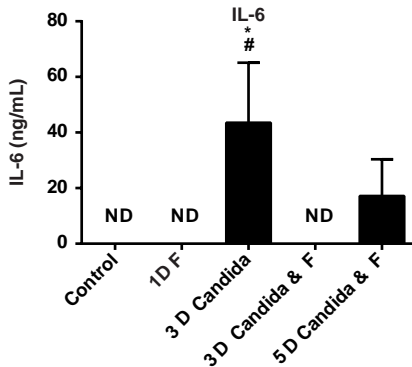


Figure 2. Plasma IL-6 concentrations following intra-amniotic *C. albicans* and/or Fluconazole treatment. For statistical analysis, undetectable values were assigned an arbitrary value of 1 pg/mL. * $P \leq 0.05$ vs. control; # $P \leq 0.05$ vs. 3D *C. albicans*/Fluconazole.

Structural analysis of the brain

Qualitative analysis of H&E stained sections revealed no evidence of structural changes including intraventricular hemorrhages and cystic lesions in any of the experimental groups. However, the brains of *C. albicans*-exposed animals had increased cell density in the white matter which primarily consisted of glial cells and blood-derived macrophages, which cannot be distinguished from microglia (Figure 3). These gliotic lesions were primarily present in the gyral crests of the white matter. In addition, animals with gliosis showed marked vasodilatation and hyperemia (Figure 3D). These inflammatory alterations were more pronounced in the 5D *C. albicans*/Fluconazole-treated group (Figure 3 C-D) compared to the 3D *C. albicans*-exposed (data not shown) and the control animals (Figure 3 A-B). Interestingly, no inflammatory alterations were detected in the subcortical white matter (Figure 3C).

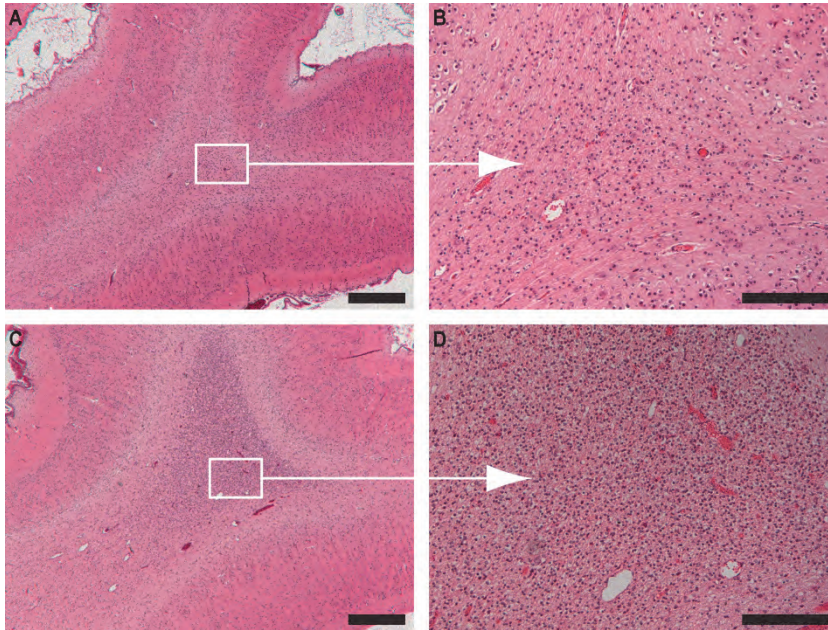


Figure 3 H&E stained brain sections of control animals and 5D *C. albicans*/Fluconazole-treated animals. Control animals (A,B) showed no to mild gliosis and the 5D *C. albicans*/Fluconazole-treated animals (C,D) showed severe gliotic foci in white matter of the gyral crest. A and C at 25x magnification (scale bar = 500 μ m) and B and D at 200x magnification (scale bar = 100 μ m).

This finding is substantiated by an increase of IBA-1 immunoreactivity in the white matter after intra-amniotic exposure to *C. albicans* irrespective of Fluconazole treatment compared to control group and/or 1D Fluconazole group (Figure 4A-D). Immunoreactivity of CD68, representative of activated microglia²⁴, was increased in the 5D *C. albicans*/Fluconazole group compared to controls (Figure 4 E-H). No significant changes in IBA-1 immunoreactivity in the periventricular white matter were observed. However, in 17% (1/6) of the 3D *C. albicans* animals and 60% (3/5) of the 5D *C. albicans*/Fluconazole animals, foci containing CD68 positive cells were found in the periventricular white matter (data not shown). Besides an increase in IBA-1 immunoreactivity, an increase of the astrocyte marker glial fibrillary acidic protein (GFAP) was found in the hippocampus after intra-amniotic exposure to *C. albicans* compared to the control group and/or 1D Fluconazole group (Figure 5). Administration of Fluconazole irrespective of pre-exposure to *C. albicans* did not induce changes in IBA-1 or GFAP immunoreactivity.

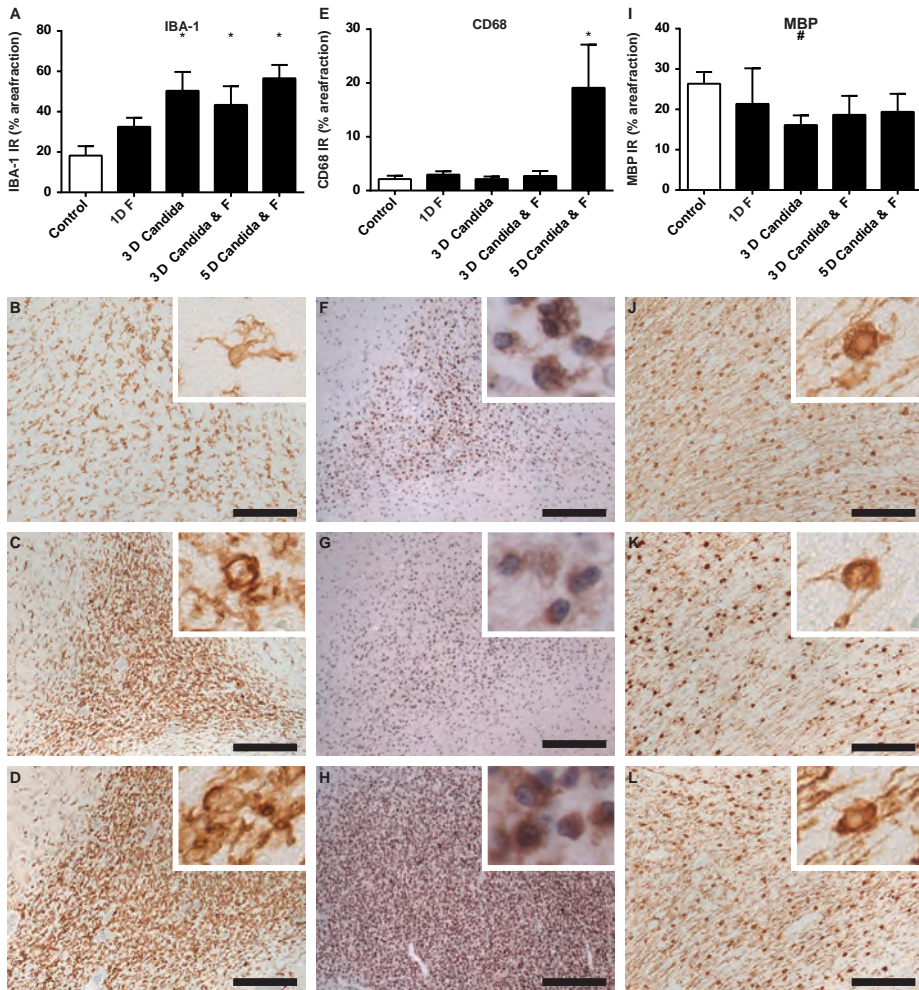


Figure 4 Antenatal exposure to *C. albicans* caused microglia activation and subsequent myelin disturbances in the white matter.

Significant increase (*, $p < 0.05$) of the area fraction of IBA-1 (A-D) immunoreactivity (IR) was observed in all *C. albicans*-exposed animals compared to controls irrespective of Fluconazole (F) treatment. A decrease of MBP IR (E-H) was observed which did not reach significance (#, $p < 0.1$) in the 3D *C. albicans*-exposed group compared to the controls irrespective of Fluconazole treatment. Representative figures show that the area fraction of the IBA-1 IR is higher in the 3D *C. albicans* group (C) and 5D *C. albicans*/Fluconazole-treated group (D) compared to the control group (B). Morphological analysis showed a higher density of amoeboid microglia present in the white matter after *C. albicans* exposure (inserts). In these regions with microglia activation, severe myelin disturbances and loss of myelin fibers were found in the 3D *C. albicans*-exposed (G) and the 5D *C. albicans*/Fluconazole-treated (H) group. Figures of the 3D *C. albicans*/Fluconazole-treated group are not depicted here. Images taken at 100x magnification (insert at 400x magnification), scale bar = 500 μm.

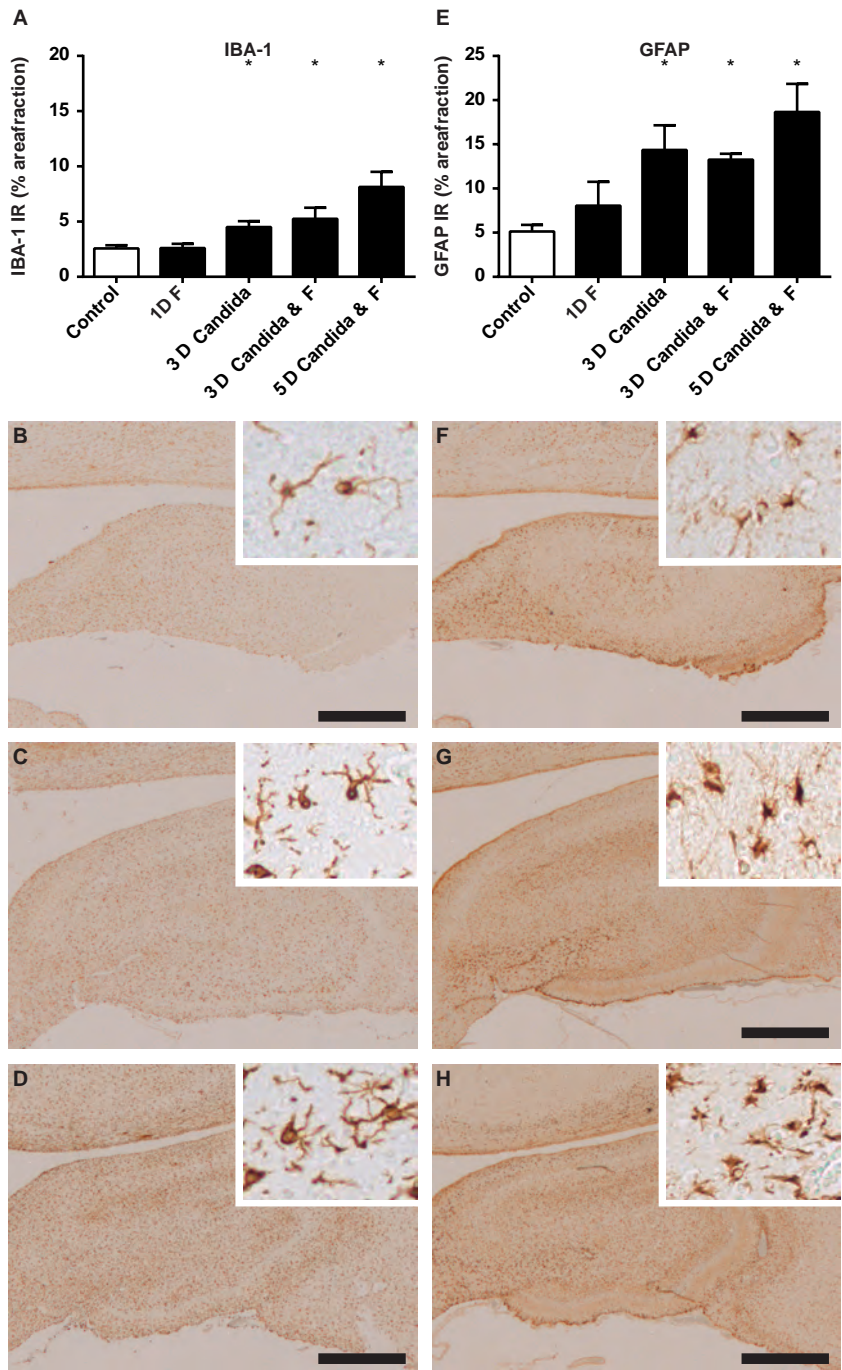


Figure 5 Antenatal exposure to *Candida albicans* caused microglial (IBA-1) and astrocyte (GFAP) activation in the hippocampus.

A significant increase (*, $p < 0.05$) of the area fraction of IBA-1 (A-D) and GFAP (E-H) immunoreactivity (IR) was observed in all *C. albicans*-exposed animals compared to controls irrespective of Fluconazole (F) treatment (A,E). No significant changes were found in the Fluconazole treated animals compared to controls. Representative figures show that the area fraction of the IBA-1 IR and GFAP IR was higher in the 3D *C. albicans* group (C,G) and 5D *C. albicans*/Fluconazole-treated group (D,H) compared to the control group (B,F). Images at 20x magnification (inserts at 200x magnification), scale bar = 100 μ m.

Antenatal exposure to C. albicans caused focal white matter disturbances with loss of mature oligodendrocytes.

There was reduced MBP immunoreactivity in regions with overt microglial activation. In particular, myelin was significantly disturbed in 5D *C. albicans*-exposed animals; in comparison to no myelin disturbances in control animals (Figure 4 I-L). The mature oligodendrocytes had severely disturbed and loss of myelin fibres in these areas of injury. The apparent loss of MBP IR prompted us to further investigate the ratio between mature and total oligodendroglial lineage cells. Double staining for CNPase and Olig2 revealed a decrease of CNPase positive cells whereas the number of Olig2 positive cells remained identical, thereby resulting in a decreased ratio of mature versus total oligodendrocytes (Figure 6 A-C).

Antenatal exposure to C. albicans did not result in neuronal degeneration

In all experimental groups no Fluoro-Jade C positive neurons were detected throughout the brain sections (data not shown).

MPO and CD3

MPO- and CD3-positive cells were sporadically observed in the cerebral vasculature of the choroid plexus and meninges. No MPO- and CD3-positive cells were found in the cerebral white matter. No differences were seen between all experimental groups (supplementary figure 1).

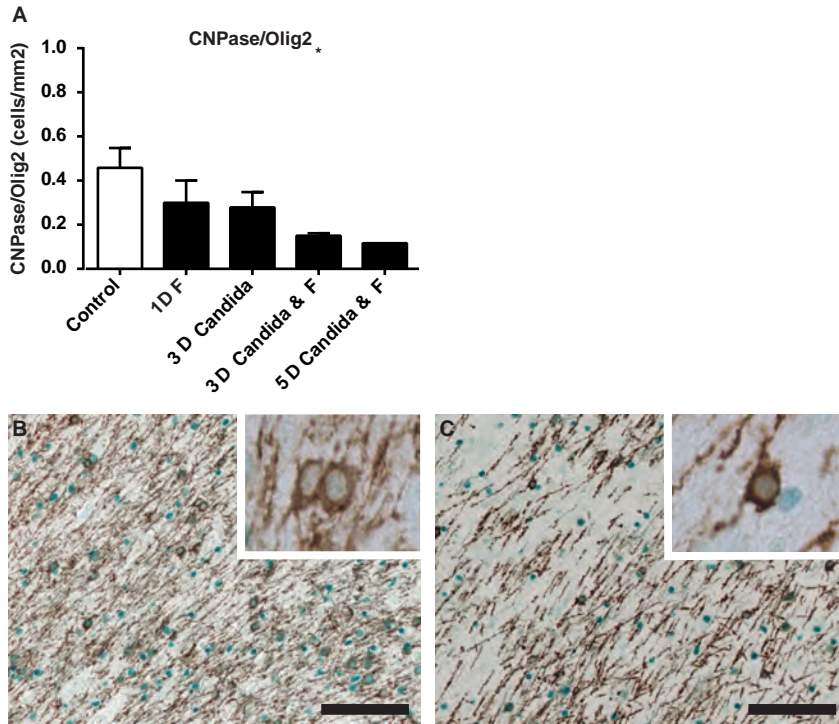


Figure 6 Myelin disturbances are accompanied by a loss of mature oligodendrocytes.

A significant decrease (*, $p < 0.05$) of the ratio between CNPase positive mature oligodendrocytes and total number of cells of oligodendroglial lineage (Olig2 positive) was observed following 3D *C. Albicans* with Fluconazole (F) treatment and 5D *C. albicans*-exposed groups with Fluconazole treatment compared to controls. (A) Graphical representation of the CNPase/Olig2 ration; (B-C) representative figures of CNPase/Olig2 double stain in (B) controls and (C) 5D *C. Albicans*-exposed animals indicating an apparent loss of CNPase positive cells. Images taken at 200x magnification (insert at 400x magnification), scale bar = 100 μ m.

Ki67 and caspase 3

An increase in Ki67 and caspase-3 positive cells was found in the white matter and hippocampus of the 5D *C. albicans*/Fluconazole group compared to controls (Figure 7 A-H, Table 2). Moreover, in the periventricular white matter, an increase in caspase-3 positive cells was observed.

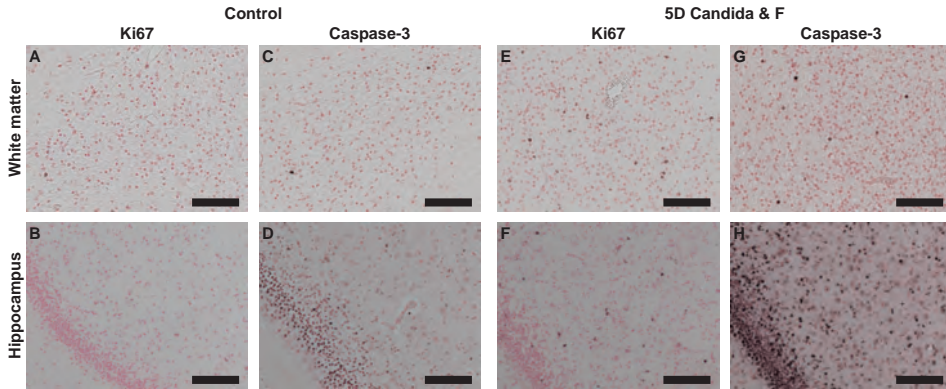


Figure 7 Antenatal exposure to *Candida albicans* resulted in increased proliferation and apoptosis in the subcortical white matter and hippocampus.

A significant increase (*, $p < 0.05$) of Ki67 and caspase-3 positive cells were observed following 5D *C. albicans* with Fluconazole treatment in the white matter (E,G) and hippocampus (F,H) compared to controls (A,B,C,D). Images taken at 200x magnification, scale bar = 100 μm .

Table 2 Ki67 and Caspase-3 positive cells.

	Control	1D Fluconazole	3D <i>C. albicans</i>	3D <i>C. albicans</i> and Fluconazole	5D <i>C. albicans</i> and Fluconazole
Ki67 + cells / mm^2					
White matter	11.16 (7.21)	41.58 (38.68)	21.85 (16.40)	54.69 (70.95)	55.99 (31.10) *
Periventricular white matter	7.585 (9.14)	14.41 (11.22)	17.61 (14.20)	8.597 (10.74)	20.90 (18.87)
Hippocampus	5.58 (4.16)	16.75 (13.61)	22.15 (27.54)	13.07 (12.98)	33.43 (27.00) *
Caspase3 + cells / mm^2					
White matter	12.80 (13.60)	14.08 (11.12)	23.68 (33.21)	16.87 (6.76)	40.22 (22.58) *
Periventricular white matter	26.14 (11.40)	35.42 (24.41)	93.44 (110.20)	46.44 (29.12)	155.10 (22.36) *
Hippocampus	364.3 (175.30)	264.4 (19.56)	443.2 (198.80)	313.4 (117.00)	726.30 (317.90) *

Ki67 and Caspase-3 positive cells expressed as mean (\pm SD). * < 0.05 versus control

DISCUSSION

The main finding of this study is that 3 days of intra-amniotic exposure to *C. albicans* resulted in increased microglial and astrocyte activation with subsequent focal white matter disturbances in the preterm ovine brain. At this stage, *C. albicans* positive CSF cultures were detected without *C. albicans*-positive immunostainings of the brain. This

may indicate that cerebral inflammation and injury in the acute phase after intra-amniotic *C. albicans* delivery is not caused by invasion of *C. albicans* in the brain parenchyma.^{25, 26} This concept is supported by the absence of neutrophils in the brain parenchyma since neutrophils are known to aggravate disruption of the blood-brain barrier promoting further invasion of *C. albicans* in the brain.²⁵ However, we cannot rule out the possibility that low titers invaded the brain, since the initial phase of *C. albicans* invasion does not require a disruption of the blood-brain barrier and such low fungal burden of the brain cannot be detected by histology or immunofluorescence.^{15, 25}

Since *C. albicans* was not detected in the fetal brain, we considered that severe systemic immune activation, as shown in the present and previous studies^{16, 17}, was (primarily) responsible for the acute changes observed in the fetal brain 3 days post *C. albicans* infection of the amniotic cavity for the following reasons: Pro-inflammatory cytokines, such as IL-6, contribute to activation of the endothelium of the blood-brain barrier, which in turn activate adjacent microglia and astrocytes, ultimately resulting in a cerebral inflammatory response.²⁷⁻³¹ In line with this, systemic inflammation is known to be causally involved in the induction of white matter injury - as seen in our model.^{32, 33} In addition, IL-6 was shown to be involved in the inhibition of neurogenesis and the development of hyper-excitable neurological conditions including epilepsy, psychoses, anxiety and autism spectrum disorders in experimental models.^{28, 34}

Notably, increased circulatory IL-6 levels were not always paralleled by positive blood cultures in our study. This finding can be explained by earlier studies in which the presence of a pro-inflammatory trigger in the amniotic fluid caused systemic inflammation without migration of microorganisms or their components into the fetal circulation.^{35, 36}

Intra-amniotic *C. albicans* caused a heterogeneous pattern of cerebral immune activation and microgliosis. Moreover, microglial activation, as identified by immunohistochemical analysis of CD68, was strongly associated with white matter injury within these specific regions. An identical pattern of systemic immune activation, neuroinflammation and concomitant structural injury was found following intra-amniotic delivery of endotoxin in the same animal model.^{21, 37-39} This indicates that irrespective of the inflammatory trigger responsible for the onset of the fetal inflammatory response, inflammation as such, and not the stimulus of inflammation, appears to be the most important factor in inflammation-induced fetal brain injury within the first days after induction of chorioamnionitis.

White matter injury can occur as a result of activation of the cerebral immune system, characterized by activated microglia and astrocytes, which produce cytokines, free radicals, nitric oxide, and excitotoxic amino acids, all of which are detrimental to the survival of oligodendrocytes.^{7, 40} The reduced CNPase/Olig2 ratio indicates that white matter injury in our model was most likely the result of death of mature oligodendrocytes. This loss was

paralleled by increased proliferation of oligodendrocyte progenitors, potentially as compensation for the observed reduction of mature oligodendrocytes.^{41, 42} It is tempting to speculate that these progenitors might fail to mature into functional myelin producing oligodendrocyte since this phenotype has previously been reported in experimental models of perinatal stress⁴³⁻⁴⁵ and in human neonates with periventricular leukomalacia.⁴¹ Finally, such oligodendrocyte maturation arrest correlates with motor deficits characteristic for cerebral palsy.^{40, 45, 46} In conjunction with white matter injury, grey matter injury plays an independent role in neurodevelopmental outcome.³⁵ However, in our study no degenerative neurons were observed.^{22, 23} In addition, cerebral palsy also comprises cognitive impairments^{7, 8} for which we analyzed the hippocampus in which a similar inflammatory response was observed which is consistent with our previous results.^{21, 39}

We previously reported that intra-amniotic *C. albicans* rapidly colonizes the amniotic fluid, causing inflammation of the fetal skin and lungs, and progresses into a fetal systemic inflammatory response causing fetal death within 5 days after exposure.^{16, 17} In the present study we show that Fluconazole administration eradicated *C. albicans* from the CSF and reduced systemic inflammation. Importantly, reduction of the fungal burden and inhibition of systemic immune activation following Fluconazole treatment was not accompanied by attenuation of cerebral inflammation and injury. Multiple studies have demonstrated that cerebral inflammation acquired during early fetal development continues postnatally and might even persist into adulthood.^{47, 48} Therefore, we consider that the observed cerebral inflammatory response following intra-amniotic *C. albicans* exposure is a persistent reaction that is initiated by peripheral immune activation. Although Fluconazole treatment did not protect against structural cerebral injury in this acute phase, the observed inhibition of systemic immune activation might be clinically relevant, since prolonged and aberrant systemic immune activation are known to induce blood-brain barrier disruption, thereby facilitating cerebral invasion of *C. albicans* and substantially aggravating cerebral injury resulting in increased morbidity and mortality.²⁶ Collectively, previous and current findings suggest that timing of infection and the start of antifungal treatment regimen are important in the initiation, maintenance, and possible resolution of neuroinflammation and structural injury in the fetal brain. The current study provides important insight in the sequelae of events that contribute to induction of adverse outcomes of the premature brain following intra-amniotic *C. albicans* infection. In addition, this study points out that Fluconazole treatment should be started immediately after intra-amniotic exposure to *C. albicans* considering the narrow therapeutic time window to reduce morbidity and mortality. Therefore, further research should focus on timing, frequency and dosing of antifungals in the presence or absence of immunomodulatory treatment.⁴⁹

Chapter 4

Fluconazole treatment was not associated with adverse effects in our study, which is in line with the findings of Maneenil and colleagues in the same model¹⁶ and studies demonstrating that teratogenic effects of Fluconazole are exclusively seen when used in the first trimester at higher doses and after multiple gifts.⁵⁰ The administered Fluconazole dose in this study mimics the amount used in systemic neonatal candidiasis in which no adverse long-term brain pathology and behavioral deficits are currently described.⁵⁰ In addition, clinical *in utero* administration of Fluconazole prolonged pregnancy in two cases of intra-amniotic *C. albicans* infection without adverse treatment effects on the fetus.¹³

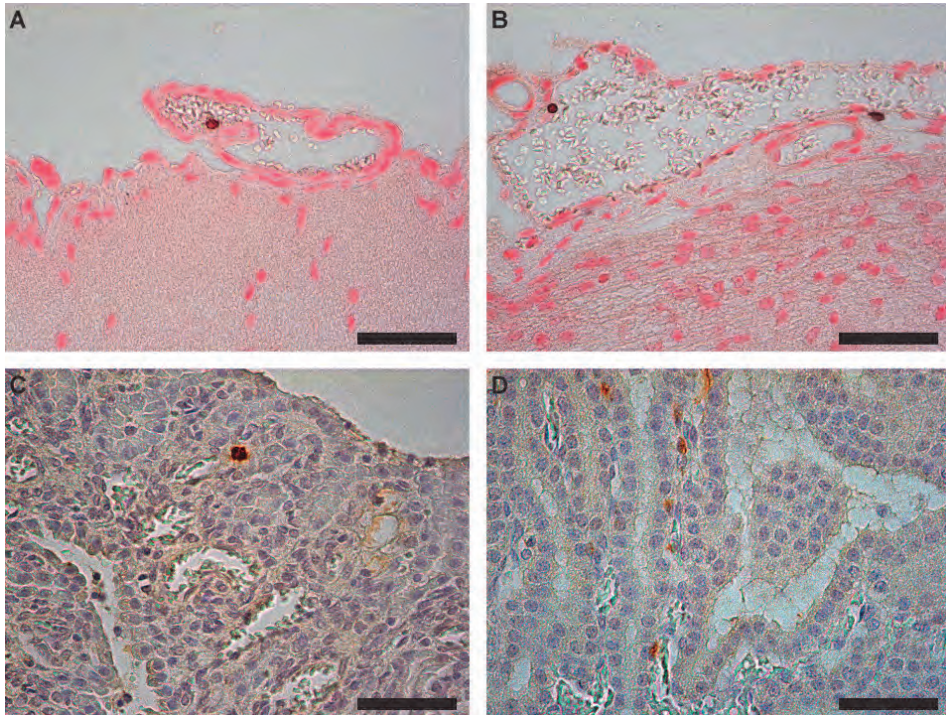
We note that our study has several limitations. We only tested one dose and frequency of Fluconazole administration. In addition, we were not able to correlate histological changes to functional neurological outcomes. However, several studies previously established an association between endotoxin-induced chorioamnionitis and functional EEG changes.^{44, 51, 52}

Conclusion

We have shown that intra-amniotic exposure to *C. albicans* results in an acute systemic and neuroinflammatory response with concomitant white matter injury. Although systemic immune activation was significantly inhibited following Fluconazole treatment, modulation of the cerebral inflammatory response and prevention of concomitant white matter injury was not found.

This study forms the essential basis for follow up studies in which the timing, frequency and dosing of antifungal treatment must be explored and alternative/additional treatment options including immunomodulatory interventions can be tested.

SUPPLEMENTARY FIGURES



Supplementary figure 1 Antenatal exposure to *C. albicans* does not result in infiltration of peripheral immune cells.

(A-B) representative pictures of CD3 positive T lymphocytes in the meninges of (A) controls and (B) 5D *C. Albicans* /Fluconazole animals. (C-D) representative pictures of MPO positive neutrophils in the choroid plexus of (C) controls and (D) 5D *C. Albicans* /Fluconazole animals.

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Chapter 4

47. Fleiss, B. & Gressens, P. Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *The Lancet. Neurology* **11**, 556-566 (2012).
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5

General Discussion

Injury of the developing brain has been strongly correlated with morbidity and mortality in premature and full-term infants, resulting in an enormous physical, psychological and economic burden.^{1, 2}

Both intra-uterine infection and hypoxia-ischemia (HI) are major causes of injury of the preterm brain. Although different in etiology, these conditions initiate a detrimental inflammatory response in the brain, leading to structural injury and subsequent dysfunction of the fetal brain.

Currently, treatment strategies are limited and mostly focused on HI in term infants, thereby excluding a large group of patients, indicating that novel therapeutic strategies are urgently warranted.

In this thesis, we assessed novel therapeutic strategies for injury of the preterm brain caused by either global hypoxia-ischemia or intra-uterine infection. Therefore, we postulated that therapies for hypoxia-ischemia-induced injury of the preterm brain should have both immune-modulatory and regenerative potential. These requirements are met by stromal cells. Furthermore, we assessed antimicrobial treatment as treatment strategy for intra-uterine infection-induced injury of the preterm brain.

We chose to test our therapeutic interventions in ovine models of global HI or intra-uterine infection since ovine developmental biology closely resembles human developmental biology and adequately mimics the etiology of brain injury.³⁻⁵ Moreover, the long gestational period (~147 days) enables us to perform detailed analyses of developmental processes at specific points during gestation. Furthermore, the size of the fetus allows instrumentation, continuous *in utero* registration of vital parameters with strong relevance and predictive values⁵⁻⁷ (i.e. blood pressure, electroencephalography, and electrocardiography), and application of intra-uterine treatment strategies.

CELL-BASED THERAPIES

Cell-based interventions have shown great promise as a therapeutic strategy for injury to the (neonatal) central nervous system.⁸⁻¹⁶ In previous studies we have shown that global HI injury of the preterm brain is mediated by a two-stepped inflammatory response in which the first inflammatory hit comprises a microglia-mediated local neuroinflammatory response followed by a systemic immune response, mediated by spleen-derived immune cells.³ Systemic administration of mesenchymal stromal cells (MSCs), before initiation of systemic immune activation, protected the preterm brain through reduction of microglial proliferation, induction of T cell tolerance, and reduction of cerebral invasion by peripheral immune-effector cells.¹⁷

However, clinical administration of cell-based therapies during early reperfusion mandates availability of large numbers of cells that can readily be administered, excluding the possibility of autologous transplantation.

Multipotent Adult Progenitor Cells (MAPCs) are adherent bone marrow-derived cells of an earlier developmental stage than MSC that have a high expansion potential with stable genetics.^{18, 19} Furthermore, their immunological properties make it possible to use them as a universal allogeneic donor.^{18, 20, 21} Moreover, compared to MSCs, MAPC cells have stronger anti-inflammatory and regenerative potential.²²⁻²⁷

In **chapter 2** we tested the neuroprotective potential of systemic administration of MAPC cells following global HI. In line with our earlier findings, we found that systemic administration of MAPC cells protected against structural and functional injury of the preterm brain. Interestingly, functional protection reached as far as the brainstem (i.e. baroreceptor reflex). Conservation of these functions indicates that structural protection by MAPC treatment also comprises protection of (deep) grey matter structures.

Also, in line with previous results, splenic involution, which is postulated to be caused by efflux of immune effector cells, was prevented by systemic MAPC administration. MAPC cells exert a plethora of neuroprotective functions that each engage at different time points in the pathophysiology of hypoxia-ischemia-induced injury of the preterm brain. Thus, timing of MAPC delivery is important to achieve specific treatment effects. We administered MAPC cells 1 hour and 4 days after the global HI insult. Inflammatory process including immune cell activation and recruitment are initiated almost immediately following global hypoxia-ischemia. Therefore, the first dose of MAPC cells was aimed to target immune-mediated secondary injury that is initiated during early reperfusion. A pilot study in this model demonstrated a peak in seizure burden in untreated animals at 4 days post hypoxia-ischemia. Therefore, we administered a second dose a 4 days post hypoxia-ischemia. Furthermore, a previous study demonstrated beneficial effects of systemically administered MAPC cells in a dose-dependent manner.^{25, 27}

In the current study we assessed outcomes 7 days after hypoxia-ischemia, with favorable effects of MAPC treatment. Long-term evaluation, including postnatal administration, may further enhance the clinical significance of these results. A recent clinical trial in which stroke patients received systemic treatment with MultiStem®, the clinical-grade product of MAPC cells, showed no difference in outcomes compared to placebo at 90 days.²⁸ However, the analysis at 365 days showed a substantial favorable effect of MultiStem® treatment compared to placebo. Furthermore, post-hoc analysis demonstrated that earlier treatment was more favorable at both 90 and 365 days follow-up.^{29, 30}

In the current model, we administered MAPC treatment *in utero* where pulmonary circulation is bypassed by the ductus arteriosus and the foramen ovale. Upon birth these two fetal cardiac shunts are closed due to the need for pulmonary gas exchange. This is accompanied by changes in blood flow. Systemic administration of stem cells during postnatal life poses a major risk for accumulation of cells in the pulmonary capillary bed, possibly leading to pulmonary emboli and subsequent respiratory distress. The smaller size of MAPC cells compared to MSC facilitates passage through the pulmonary capillary bed, thereby reducing the risk of pulmonary complications and increasing availability of MAPC cells in the systemic and cerebral vasculature and thus enhancing their neuroprotective effects.³¹ Furthermore, in accordance to studies demonstrating that MAPC are cleared from the bloodstream within 48 to 72 hours after administration, we did not detect MAPC in brain, spleen or lung tissue 7 days after global HI (i.e. 3 days (i.e. 3 days after the second dose of MAPC)).^{26, 32} These findings indicate that MAPC are cleared in the recipient several days after administration. This implies that potential long term side-effects of cell therapy are reduced to a minimum.

Whereas immune-modulation is a crucial component of the therapeutic effects of MAPC treatment, caution should be taken when administered to preterm infants. Due to an underdeveloped immune system and potential *in utero* immune challenges, including chorioamnionitis, preterm infants are extremely vulnerable for infections. Alterations to the fetal immune system might lead to temporary immune-suppression and the inability to combat infections.³³ Alternatively, MSCs have proven to enhance the phagocytic ability of immune cells, including neutrophils, and improve effective pathogen clearance, thereby reducing the severity of sepsis.³⁴

Transplanted MSCs exert their protective effects primarily through trophic support. This concept is supported by studies demonstrating comparable therapeutic effects of MSC-conditioned medium when compared to its cellular equivalent.^{35, 36} In addition to secreting an array of soluble factors, it has also been recognized that MSCs release large numbers of extracellular vesicles (EVs) which are involved in cellular communication as paracrine mediators and possibly transfer trophic factors between cells.

Therefore, in a study described in **chapter 3**, we investigated the therapeutic effects of intravenous administration of MSC-EVs on HI-induced injury of the preterm brain. Intravenous MSC-EV administration resulted in a reduction of electro cortical seizure burden and preservation of the baroreceptor reflex sensitivity after global HI and partially protected against structural injury of the preterm brain after global HI without reducing cerebral inflammation. In contrast to previous findings, suggesting that neuroprotection of stromal cell therapy (MSC and MAPC respectively) could, at least in part, be attributed to attenuation of neuroinflammation, MSC-EV administration did not attenuate neuroinflammation. This implicates that in addition to anti-inflammatory mechanisms, inflammation-independent mechanisms play a crucial role in the

neuroprotective potential of stromal cells. This concept is supported by several studies showing that MSC-EVs establish neuroprotection and regeneration through stimulation of angiogenesis and neurogenesis after hypoxia-ischemia.³⁷⁻⁴³ MSCs are known to secrete a number of neurotrophic and angiogenic factors, induce angiogenesis, neurogenesis and astroglial growth and activation, promote synaptogenesis and therefore enhance synaptic connections and axonal remyelination, and decrease apoptosis. Furthermore, it was recently shown in neuron cultures and mice using models of excitotoxicity that administration of MSCs or MSC-conditioned medium mediated neuroprotection through inhibition of glutamate receptor expression and function.⁴⁴

Despite the observed discrepancies in therapeutic effects between MSCs and MSC-EVs, the application of EVs offers several advantages over the administration of MSCs (table 1): (1) the potential risk of malignant transformation is greatly reduced because EVs are non-self-replicating. (2) Due to their small size, EVs are less likely to generate emboli upon intravenous administration, as may be the case with MSCs. (3) EVs can be sterilized by filtration. Thus, from a regulatory point of view, the production and especially the quality control of EV fractions for clinical treatment application are less complicated compared to a primary cells or *in vitro* expanded cells. (4) EVs can be developed independently of the original donor (as they are derived from MSCs from unrelated donors) and thus offer the opportunity to be turned into an “off-the-shelf” product. (5) EVs are easier to store, and in the future freeze-dried exosome preparations will be available.⁴⁵ This reduces the logistic burden involved in storage of stem cells in liquid nitrogen and makes the cell product readily available within the short window of therapeutic opportunity after the injurious insult. (6) EVs may more easily cross the blood-brain-barrier, possibly enhancing the protection of the penumbra where the blood-brain-barrier is still intact.

The beneficial effects of MSC treatment cannot be exclusively attributed to release of trophic factors. A study by González et al. demonstrated that macrophages and adipose-derived MSCs remain their IL-10 production only when cell-cell contact is maintained.⁴⁸ Also, stromal cells intrinsically possess immune-modulatory properties that are enhanced by a pro-inflammatory micro environment *in vivo*. The obtained EVs used for intravenous administration were obtained from unlicensed MSCs. Lacking an own metabolism, these EVs cannot modify their content, which may account for the absence of anti-inflammatory properties. However, controlled *in vitro* licensing or genetic manipulation of MSCs might enhance the anti-inflammatory potential and contribute to a targeted clinically safe cell-free therapy.⁴⁹ This type of manipulation requires a uniform homogeneous population of stromal cells with stable genetics throughout expansion and manipulations.⁴⁹ These criteria are met by MAPC cells.

Table 1

Comparison	Mesenchymal stem cells ¹	Multipotent Adult Progenitor Cells ²	Mesenchymal stem cell-derived extracellular vesicles ³
Neuroinflammation	Attenuation of inflammation (local)	Attenuation of inflammation (local)	No protection
Structural injury	White matter protection	White matter protection	No protection
Brain function	Reduced seizure burden	Reduced seizure burden	Reduced seizure burden
		Improved baroreceptor reflex sensitivity	Improved baroreceptor reflex sensitivity
Selectivity	Adjusts to <i>in vivo</i> microenvironment (licensing)	Adjusts to <i>in vivo</i> microenvironment (licensing)	Determined by <i>in vitro</i> condition of 'mother cell'
Clinical product	Heterogeneous population	Homogenous population	Sterilized by filtration
	Limited expansion potential	High expansion potential with stable genetics	Can be developed independently of the original donor
	Low immunogenicity	Low immunogenicity	
		Off-the-shelf product: MultiStem®	Readily available within time window of opportunity (i.e. 6 hours after HI)
		Readily available within time window of opportunity (i.e. 6 hours after HI)	Variability from batch to batch production

¹ Based on Jellema et al., 2013.¹⁷

² Based on Jellema & Ophelders et al., 2015.⁴⁶

³ Based on Ophelders et al., 2016.⁴⁷

In contrast to HI-induced neuroinflammation, which is a sterile inflammatory reaction in response to a single insult (hypoxia-ischemia respectively), intra-amniotic exposure to *Candida albicans* (*C. albicans*), as investigated in **chapter 4**, caused a persistent systemic immune response with a concomitant microglial and astroglial response, white matter injury, and fetal death within 5 days upon infection, without apparent invasion into the brain parenchyma. A similar pattern of cerebral injury was observed following intra-amniotic exposure to endotoxin. Intra-amniotic Fluconazole treatment was successful for eradication of the infectious trigger and temporarily reduced the systemic immune response without attenuation of the neuroinflammatory response and protection against structural injury. Nonetheless, and most importantly, single treatment promoted fetal survival. Collectively, we concluded that inflammation as such, and not the stimulus, to be the most important factor in fetal brain injury within the first days after induction of chorioamnionitis.

Our current findings suggest that timing of the (antifungal) treatment regimen is of great importance as early treatment prevented fetal demise. However, a single dose regimen temporarily reduced the systemic response and failed to prolong this reduction. Therefore, we postulate that a multiple dosing strategy will be beneficial for complete eradication of *C. albicans* and will attenuate the subsequent systemic immune response. As eradication of the fungus and (temporary) reduction of the systemic

immune response had no effects on cerebral inflammation and structural injury, these data suggest that additional immune modulatory therapy is required to protect the fetal brain.

Cell-based therapies might be such an immune-modulatory therapy. Our previously described studies on the therapeutic effects of cell-based therapies following global hypoxia-ischemia have demonstrated reduction of the detrimental cerebral and peripheral immune reaction, and prevented against white matter injury.^{17, 46, 47}

Besides neuroprotective capacities, cell-based therapies (MSC) have proven therapeutic efficacy in models of necrotizing enterocolitis (NEC) and bronchopulmonary dysplasia (BPD), which are two major complications of intra-uterine infection and subsequent preterm birth^{4, 50-52} and are correlated to adverse neurodevelopment.⁵³ Mostly inflammation-dependent mechanisms^{54, 55} are described, but effects on impaired cerebrovascular autoregulation and subsequent hypoxia-ischemia might play essential roles as well.⁵⁶ Therefore, the immune modulatory effects of MSCs may not only improve intestinal and pulmonary outcomes in NEC and BPD but may also indirectly prevent adverse neurodevelopmental outcomes by preventing secondary cerebral injury and supports a multi-organ approach through systemic administration of MSCs.

Furthermore, several studies have shown beneficial effects of MSCs in various *in vitro* and *in vivo* models of sepsis. Human MSCs have shown to attenuate NF- κ B activation and nuclear translocation during inflammatory conditions.^{57, 58} Furthermore, interferon gamma-stimulated MSCs showed broad anti-microbial effector functions.⁵⁹ *In vivo* models of sepsis confirmed the antimicrobial functions of MSCs, which reduced the number of colony-forming-units and decreased bacterial counts.⁶⁰⁻⁶² Mechanisms responsible for the antimicrobial actions of MSCs are attributed to inflammation-induced secretion of antimicrobial proteins (i.e. bacterial defensins, LL-37, lipocalin-3, hepcidin, and keratinocyte growth factor, improvement of phagocytosis activity of blood monocytes, increased circulating neutrophil counts and enhanced neutrophil respiratory bursts.⁶³⁻⁶⁷

Neuroprotective strategies for perinatal infection

At present no established therapies to reduce inflammation-related injury of the preterm brain are available. Possible therapies include antibiotics, anti-inflammatory agents, inhibition of cell-death pathways, promotion of cellular maturation, and development, and cellular regeneration/replacement.

Antibiotic treatment has been tested extensively, but demonstrated that prophylactic therapy increased the risk of neonatal death and neurodevelopmental disability. Postnatal treatment with tetracycline (minocycline) treatment has been associated with improved neurodevelopmental outcomes in animal models of intra-uterine

inflammation. However, due to the risk of disturbed bone and tooth enamel formation, tetracyclines are contraindicated in the current setting of inflammation induced injury of the preterm brain.⁶⁸

Antenatal maternal glucocorticoid administration is routine treatment when preterm labor is pending as they enhance lung maturation, thereby preventing acute neonatal morbidity and mortality.⁶⁹ Mixed reports have been published on the effects of glucocorticoid treatment on neurodevelopmental outcomes. A report by Kuypers et al. demonstrated that prophylactic glucocorticoid treatment prevented adverse neurodevelopmental outcomes, whereas treatment after the onset of intra-uterine inflammation exacerbated injury.⁶ Collectively, these data suggest that glucocorticoid therapy is not beneficial when infection/inflammation is established.

Reactive oxygen species play essential roles in the pathogenesis of brain injury.^{70, 71} Melatonin, a naturally occurring circadian hormone with antioxidant and anti-inflammatory properties⁷², has proven neuroprotective properties in a piglet model of perinatal asphyxia caused by both hypoxia-ischemia and intra-uterine inflammation.⁷³ Moreover, it is already widely used clinically.⁷⁴ Erythropoietin (EPO), which is a hematopoietic hormone that regulates erythrocyte production, has shown to possess anti-inflammatory, anti-oxidant, anti-apoptotic, and neurotrophic properties.⁷⁵ Increasing evidence arises on the neuroprotective properties of EPO in inflammation-induced brain injury. Continuous administration of recombinant EPO during the early recovery phase in an ovine model of global hypoxia-ischemia was neuroprotective and improved electrophysiological and cerebrovascular recovery, in association with reduced apoptosis and inflammation.⁷⁶ Moreover, a recent clinical study by Song et al. demonstrated that repeated administration of a low-dose rhEPO for two weeks significantly reduced the incidence of brain injury, including intra cranial hemorrhage and periventricular leukomalacia, in preterm infants. Besides neuroprotection, rhEPO administration decreased the risk for NEC and sepsis, thereby indicating its wide-spread protective effects.⁷⁷

FUTURE DIRECTIONS

In this thesis we have demonstrated that immune modulation protects the preterm brain from injury and dysfunction after *in utero* adverse events (e.g. intra-amniotic infection and hypoxia-ischemia). Within the context of immune modulation, future research should focus on immunological interplay between the central nervous system and the peripheral immune system.

Dual role of microglia / macrophages

As described before, microglia are among the first responders to injury of the preterm brain. Moreover, they are among the most potent modulators of CNS repair and regeneration. However, they have dualistic roles in the battle for neurological recovery. On the one hand, microglia activation promotes brain recovery by clearing cell debris, resolving local inflammation, and releasing a plethora of trophic factors. On the other hand, microglia activation hinders CNS repair and contributes to expansion of tissue damage. These opposite functions are reflected in the acquisition of distinct phenotypes in response to different micro environmental signals. Classically activated M1 microglia typically release destructive pro-inflammatory mediators. In contrast, alternatively activated M2 phenotypes release numerous protective/ trophic factors. In particular the M2 phenotype is known to promote regenerative processes including neurogenesis, axonal remodeling, angiogenesis, oligodendrogenesis, and remyelination. Considering these adaptive functions, treatment strategies should be aimed at modulation of neuroinflammation instead of suppression. MSCs and MAPC cells have proven capacities to drive microglia polarization *in vitro* and *in vivo*.^{24, 78} However, the effects on microglia/macrophage polarization in our models remain to be elucidated and proper diagnosis of intra-uterine infection and/or hypoxia-ischemia without apparent life threatening fetal distress is mandatory.

Recent studies in CNS injury models show that the majority of newly recruited microglia/macrophages at the site of injury express M2 signature genes whereas microglia expressing M1 genes dominate the site of injury about 1 week after injury.⁷⁹⁻⁸¹ This phenotype switch from M2-dominant to M1-dominant may result from a M2-to-M1 conversion within activated microglia, as well as the recruitment of pro-inflammatory M1 microglia/macrophages to the site of injury.⁸² In addition, although both white and grey matter exhibit M2-to-M1 switch after injury, the temporal dynamics of the switch differ between the two types of tissue and more prominent phenotypic changes occur in white matter.⁸² The temporal dynamics of microglia phenotypical switch indicate that timing of treatment is of crucial importance since prevention of M1 microglia as the prevailing phenotype might act as therapeutic target. Moreover, *in vivo* studies using bone marrow chimeras to distinguish microglia from bone marrow-derived macrophages have confirmed that resident microglia and bone marrow-derived macrophages are both involved in phenotype switching.^{83, 84} Moreover, studies have shown that monocytes can be primed towards an immune modulatory M2 phenotype through exposure to mesenchymal stromal cells prior licensed to exert anti-inflammatory functions.^{85, 86} As such, we postulate a role for monocytes as therapeutic target. We hypothesize that *in vivo* education of monocytes by MSCs or MSC-EVs and subsequent differentiation into alternatively activated macrophages might contribute to attenuation of neuroinflammation and promote regeneration. Within this concept, the

spleen, as a reservoir for immune-effector cells, is of crucial importance since splenic monocytes, in contrast to bone marrow and peripheral monocytes, have demonstrated to generate alternatively activated macrophages following exposure to MSCs.⁸⁷

Furthermore, targeting peripheral immune effector cells to exert neuroprotective functions would implicate that homing of stem cells in the cerebral vasculature and parenchyma is no prerequisite for neuroprotective actions and justifies intravenous administration, as performed in this thesis. Collectively, this paves the road for modulation of the fetal immune system following *in utero* ischemic compromise through maternal administration of stem cells. Recently, maternally administered MSCs modulated the maternal immune system and the fetal immune response following intra-amniotic endotoxin exposure. Interestingly, these maternally administered MSCs protected against preterm birth, brain injury and motor deficits.⁸⁸

As proposed in chapter 3, enhancing the anti-inflammatory potential of MSC-EVs through inflammatory licensing might improve their therapeutic efficacy. Also, understanding the temporal dynamics of injury and repair might reveal specific phases that require enhancement of processes other than anti-inflammatory, such as angiogenesis. Generation of EVs that specifically target these processes can contribute to neuroprotection. Moreover, as suggested in chapter 4, exerting a synergistic approach in which cell-based therapies are combined with therapeutic approaches, such as EPO, melatonin, glucocorticoids, and hypothermia, might improve neurodevelopmental outcomes. Such a synergy has been demonstrated in an animal model of BPD where combined treatment with MSCs and erythropoietin resulted in more promotion of lung repair compared to MSCs or EPO treatment alone.⁸⁹

In conclusion, the work described in this thesis demonstrated the promising effects of immune-modulatory therapies in the protection of the preterm brain after intra-amniotic infection and hypoxia-ischemia, which all activate the immune system. Temporal dynamics of peripheral immune effector cell mobilization and subsequent cerebral infiltration might therefore provide information crucial for timing of cell-based, cell-derived or anti-microbial therapies. Furthermore, the potential clinical impact and mechanisms of action of these promising therapies needs to be further assessed in long-term follow-up studies in appropriate animal models.

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Summary

Brain damage is a major contributor to mortality and morbidity in preterm infants and is mostly caused by lack of oxygen (hypoxia-ischemia) and / or intra-amniotic infection (chorioamnionitis). At present there is no cure for brain damage in this vulnerable group of children and current therapies are aimed at treatment of symptoms and stabilizing vital functions. It is, therefore, important that new treatment strategies be developed for the protection of the premature brain.

The aim of this thesis was to test new therapies for brain damage caused by oxygen deprivation or treat an amniotic fluid infection in preterm infants.

CELL-BASED THERAPY

Systemic and local immune activation are causally involved in the pathophysiology of brain injury, regardless of its cause. However, activation of immune cells is also necessary for regeneration of injured tissue and an imbalance in these two properties leads to brain injury in preterm infants.

Therefore, we hypothesized that therapeutic strategies should possess (1) anti-inflammatory and (2) regenerative properties. Stem cells meet both of these characteristics.

In **chapter 2** we investigated the neuroprotective effects of Multipotent Adult Progenitor Cells (MAPCs), which are bone marrow-derived stromal cells with strong anti-inflammatory and regenerative capabilities, in our preclinical ovine model of hypoxia-ischemia.

Administration of MAPCs improved brain function by reducing the number and duration of convulsions. Also, blood pressure regulation in the brainstem was protected.

In addition to functional protection, administration of MAPCs also resulted in a reduced neuroinflammatory response and prevented damage to the cerebral white matter, which is characteristic for brain injury in premature babies).

The mechanisms by which bone marrow-derived stromal cells exert their immune modulatory actions are unclear, but paracrine mechanisms, mediated in part through release of extracellular vesicles (EVs) are suggested to be responsible for these effects.

In **chapter 3**, we therefore, investigated the protective effects of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in the same preclinical model of preterm hypoxic-ischemic brain injury. Systemic administration of MSC-EVs improved brain function by reducing the total number and duration of seizures, and by preserving baroreceptor reflex sensitivity. These functional protections were associated with a tendency to prevent hypomyelination. Cerebral inflammation remained unaffected by the MSC-EV treatment.

ANTIMICROBIAL TREATMENT

In **chapter 4** we investigated the effects of intra-amniotic *Candida albicans* (*C. albicans*) infection on the preterm brain. Furthermore, we investigated the effects of reducing the fungal load by intra-amniotic antifungal treatment with Fluconazole.

Intra-amniotic exposure to *C. albicans* caused a severe systemic inflammatory response, illustrated by a robust increase of plasma IL-6 concentrations. Cerebrospinal fluid cultures were positive for *Candida albicans* in the majority of the group of 3 day *C. albicans*-exposed animals whereas no positive cultures were present in the group of 5 day *C. albicans*-exposed and Fluconazole treated animals. *C. albicans* was not detected in the brain parenchyma. A neuroinflammatory response in the hippocampus and white matter was however detected which was characterized by increased microglial and astrocyte activation. The neuroinflammatory changes were accompanied by structural white matter injury. Intra-amniotic Fluconazole reduced fetal mortality, but did not attenuate either neuroinflammation or white matter injury.

In summary, this thesis demonstrated that modulation, rather than attenuation, of immune reactions with cell-based or anti-microbial treatment protected against injury of the preterm brain following global hypoxia-ischemia or intra-uterine infection. The therapies tested in this thesis show great promise and will form the basis of additional research and clinical trials, leading to improved neurodevelopmental outcomes after HI or intra-uterine infection.

Samenvatting

Hersenschade heeft een belangrijke bijdrage aan de sterfte en morbiditeit bij vroeggeboren kinderen en wordt voornamelijk veroorzaakt door zuurstoftekort (hypoxie-ischemie) en / of een vruchtwaterinfectie (chorioamnionitis). Op dit moment is er geen behandeling voor hersenschade in deze kwetsbare groep kinderen. Huidige therapieën zijn dan ook gericht op het behandelen van de symptomen en het stabiliseren van de vitale functies. Het is daarom belangrijk dat nieuwe behandelingsstrategieën voor de bescherming van het brein van vroeggeborenen worden ontwikkeld.

Het doel van dit proefschrift was het testen van nieuwe therapieën om hersenschade, veroorzaakt door zuurstoftekort of een vruchtwaterinfectie, in vroeggeboren kinderen te behandelen.

STAMCEL THERAPIE

Activatie van immuun cellen zowel in als buiten het brein is betrokken bij de ontwikkeling van hersenschade. Echter, deze ontstekingsreactie is ook nodig om beschadigd weefsel te herstellen. Een dysbalans in deze twee eigenschappen leidt ertoe dat hersenschade in vroeggeboren kinderen niet herstelt.

Daarom hypothetiseerden wij dat een therapie de (1) de ontstekingsreactie moet remmen (anti-inflammatoir) en (2) weefselherstel moet stimuleren. Stamcellen voldoen aan beide kenmerken.

In **hoofdstuk 2** onderzochten we daarom de beschermende effecten van Multipotent Adult Progenitor Cells (MAPCs) op hersenschade na zuurstof tekort. Dit onderzochten wij in een translationeel diermodel voor premature breinschade ontstaan door zuurstoftekort. MAPCs zijn stamcellen uit het beenmerg met sterke ontstekingsremmende en weefselherstellende capaciteiten.

Toediening van MAPCs verbeterde de hersenfunctie door het aantal en de duur van convulsies te verminderen. Tevens werd de regulatie van de bloeddruk, welke geregeld wordt vanuit de hersenstam, beschermd. Naast functionele bescherming, resulteerde toediening van MAPCs ook in een verminderde ontstekingsreactie in het brein en werd schade aan de witte stof (kenmerkend voor hersenschade in vroeggeborenen) voorkomen.

De mechanismen waarop stamcellen het immuun systeem kunnen beïnvloeden zijn grotendeels onbekend. Een voorgesteld mechanisme is door uitscheiding van extracellulaire (EVs) waarin immuun modulerende moleculen verpakt zitten. Om de bijdrage van EVs aan de beschermende effecten van stamcellen op het premature brein te onderzoeken hebben we in **hoofdstuk 3** de effecten van extracellulaire vesikels uit mesenchymale stamcellen (MSC-EV) na zuurstof tekort onderzocht in ons translationeel diermodel

Toediening van MSC-EVs verbeterde de hersenfunctie door vermindering van het totale aantal en de duur van de convulsies, en door het behoud van bloeddruk regulatie vanuit de hersenstam. Deze functionele bescherming werd geassocieerd met een neiging om witte stof schade te voorkomen voorkomen. De ontstekingsreactie in het brein werd niet verbeterd door behandeling met MSC-EVs.

ANTIMICROBIËLE BEHANDELING

In **hoofdstuk 4** onderzochten we de effecten van een vruchtwaterinfectie met *Candida albicans* (*C. albicans*) op het premature brein. Tevens onderzochten we welk effect een eenmalige toediening van anti-schimmel medicatie (Fluconazol) in het vruchtwater had op hersenschade.

Een vruchtwaterinfectie met *C. albicans* veroorzaakt een ernstige systemische ontstekingsreactie in de foetus, gekenmerkt door een sterke toename van de IL-6 concentratie in het bloed. Daarnaast werd *C. albicans* na drie dagen terug gevonden in de hersenvloeistof (cerebrospinale vloeistof) van de foetus en leidde het tot foetale dood 5 dagen na infectie. Daarnaast veroorzaakte de vruchtwaterinfectie een ontstekingsreactie in het foetale brein die gepaard ging met schade aan de witte stof.

Behandeling met Fluconazol voorkwam foetale dood en verminderde de aanwezigheid van *C. albicans* in de hersenvloeistof en het hersenweefsel 5 dagen na de infectie. Ook de ontstekingsreactie in het bloed werd tijdelijk geremd. Echter, de ontstekingsreactie en schade aan het premature brein werden niet voorkomen.

Samengevat, in dit proefschrift hebben we aangetoond dat sturing van immuun reacties, in plaats van remming, met stamcellen of antimicrobiële behandeling beschermend kunnen werken tegen hersenschade bij vroeggeboren kinderen. De geteste therapieën in dit proefschrift zijn veelbelovend en zullen de basis vormen voor toekomstig onderzoek en klinische studies leidend tot een behandeling voor hersenschade na zuurstoftekort, vruchtwaterinfecties en vroeggeboorte.

Valorization

Preterm birth is the leading cause of perinatal morbidity and mortality in developed countries. In the Netherlands 7.7% of all neonates are born preterm (less than 37 weeks gestational age) of which 20% are born extremely preterm (less than 32 weeks gestational age). Although survival after preterm birth has increased in the last decades, still a large proportion of preterm infants suffer from long term morbidity and disability, which have a tremendous impact on patients and their families.

Brain injury is the biggest contributor to mortality and morbidity in preterm infants. 25-50% of preterm infants suffer from cognitive, socialization, attentional and/or behavioral disorders, whereas, 5-10% suffer from spastic motoric deficits (e.g. cerebral palsy). Current treatment strategies are focused on limiting secondary injury after hypoxia-ischemia by hypothermia treatment in term babies or treating symptoms and stabilizing vital signs in preterm babies. Infections are treated with anti-microbial agents who are aimed at the microorganisms but not at the injured brain.

Improvement of neurodevelopmental outcomes of this vulnerable group of patients would markedly decrease the burden for this vulnerable patient group. Therefore, the aim of the current thesis was to investigate therapeutic strategies for the prevention of injury of the preterm brain caused by infectious (intra-uterine *C. albicans* infection) and sterile inflammatory triggers (global hypoxia-ischemia). For this purpose we used a translational ovine model of preterm brain injury caused by either global hypoxia-ischemia or intra-uterine infection. We used ovine fetuses since ovine fetal development and physiology are similar to the human situation. Furthermore, the size of the fetus allows for chronic instrumentations and the long gestational period enables us to study specific developmental processes in detail allowing us to apply our therapies at specific time-points.

In this thesis we demonstrated that systemic administration of Multipotent Adult Progenitor Cells (MAPCs) protected the fetal brain following global hypoxia-ischemia. Especially preterm infants suffering from global HI might benefit from MAPC treatment, since early interventions (within 6 hours following global HI) are mandated. Autologous transplantation of sufficient numbers of bone marrow stromal cells is time-consuming and exceeds the therapeutic window of opportunity. MAPC treatment has already been translated into a FDA-approved clinical-grade product, named MultiStem® (Athersys Inc.), and is therefore already available “off-the-shelf”.

Currently, MultiStem® is tested extensively in phase 2 clinical trials of ischemic stroke, and preclinical trials traumatic brain injury, multiple sclerosis, and spinal cord injury. Besides neurological indications, other diseases such as cardiovascular, and inflammatory and immune conditions benefit from MAPC treatment. The results in this thesis contribute to the addition of new group of patient, namely preterm infants.

The therapeutic effects of MAPC are mostly attributed to anti-inflammatory properties. The origin of many complications of preterm birth can be narrowed down to

inflammation, both *in utero* and postnatally. Therefore, the application of MAPC might reach further than neuroprotection. Future studies will investigate the therapeutic efficacy of MAPC treatment in other neonatal diseases such as bronchopulmonary dysplasia and necrotizing enterocolitis.

Besides anti-inflammatory properties, regenerative properties are also assigned to the therapeutic potential of MAPC cells. This paves the way for MAPC treatment as a secondary treatment in case of injury caused with an infectious origin, as proposed in chapter 4 in which we demonstrated that anti-microbial treatment reduced systemic inflammation, but could not protect against injury of the preterm brain.

As the therapeutic potential of stem cells is mostly achieved by secretion of paracrine factors, packed in extracellular vesicles, we assessed the therapeutic potential of systemic administration of these vesicles, rather than administration of the complete cell product. Administration of EVs poses advantages over administration of cells as (1) the risk of malignant transformation is greatly reduced because EVs are non-self-replicating (51). (2) Lacking an own metabolism, the EVs activity can hardly be influenced by the *in vivo* environment in patients, thus allowing for a much better characterization of their functional properties. (3) Owing to their small size EVs are less likely to generate emboli upon intravenous administration, as may be the case with MSCs. (4) In addition, EVs can be sterilized by filtration. Thus, from a regulatory point of view, the production and especially the quality control of EV fractions for clinical treatment application is less complicated than for a cellular therapeutic of *in vitro* expanded cells (52). (5) Last but not least, EVs can be developed independently of the original donor as they are derived from MSCs from unrelated donors and thus offer the opportunity to be turned into an “off-the-shelf” product. MAPC cells are ideal candidates for an off-the-shelf cell-based product and might provide the source for a superior cell-free product as well.

The ability of cells to adjust their secretome *in vivo* in reaction to their microenvironment appears to be crucial for the efficacy of cell-based therapy. Due to a lack of a metabolism, EV therapy is static, and the therapeutic properties of the EVs are determined by the *in vitro* conditions of the donor cell. Since *in vitro* conditions can be altered, creation of EVs with specific therapeutic properties is possible. Moreover, with the genetic stability of MAPCs, uniform cellular responses upon *in vitro* stimulation might be expected and reflected in the generation of EVs with specific therapeutic capacities (i.e. immune-modulatory, regenerative), that can be administered off-the-shelf at specific time-points. Determination of these time-points requires specific biomarkers, for which our translational ovine model is well-suited.

In conclusion, the therapies tested in this thesis show great promise and form the basis of additional research and clinical trials, leading to improved neurodevelopmental outcomes, and, ultimately, increased disability-free lives.

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Curriculum vitae

Daan Ophelders werd geboren op 8 juli 1983 in Venlo. Hij behaalde zijn VWO diploma in 2002 aan het Valuascollege in Venlo om vervolgens in opleiding te gaan tot operatieassistent in het Maastricht Universitair Medisch Centrum (Academisch ziekenhuis Maastricht). In 2005 behaalde hij zijn diploma en hij heeft tot 2011 als operatieassistent gewerkt in het Maastricht Universitair Medisch. Gedurende zijn werkzaamheden als operatieassistent is hij in 2006 gestart met de Bachelor studie Moleculaire Levenswetenschappen aan de Universiteit Maastricht gevolgd door de Master Molecular Life Science met specialisatie oncologie en ontwikkelingsbiologie waarvoor hij in 2011 afstudeerde bij de afdeling Kindergeneeskunde onder de begeleiding van Dr. Reint Jellema. Zijn afstudeerstage heeft hij gecontinueerd als onderzoeksassistent en vervolgens een promotietraject onder de supervisie van Dr. Tim Wolfs, Dr. Reint Jellema en Prof. Dr. Boris Kramer. De resultaten van het promotieonderzoek zijn in dit proefschrift beschreven. In januari 2017 is hij begonnen als post-doctoraal onderzoeker bij de afdeling Kindergeneeskunde in het MUMC+ onder de supervisie van Dr. Tim Wolfs. Daan Ophelders woont sinds 2010 samen met Yill Raduzzi in Heerlen.

Daan Ophelders was born on July 8, 1983 in Venlo, the Netherlands. In 2002 he graduated from secondary school (Atheneum) at the Valluascollege in Venlo. Subsequently he moved to Maastricht to go into training to become a surgical assistant at the Maastricht University Medical Center (Academic Hospital Maastricht). In 2005 he graduated from his training and he remained working in Maastricht until 2011. During his work in the surgical department, he started in 2006 with the Bachelor study Molecular Life Sciences at Maastricht University, followed by the Master Molecular Life Science with specialization in oncology and developmental biology. In 2011 he obtained his Master's degree after a senior internship at the Department of Pediatrics under the guidance of Dr. Reint Jellema. After graduation for his Master's degree, he started working at the Department of Pediatrics as a research assistant, followed by a PhD trajectory under the supervision of Dr. Tim Wolfs, Dr. Reint Jellema and Prof. Dr. Boris Kramer. The results of his doctoral research described in this thesis. Since January 2017 he works as a post-doctoral researcher in the Department of Pediatrics at the Maastricht University Medical Center+ under the supervision of Dr. Tim Wolf. Daan Ophelders lives together with Yill Raduzzi in Heerlen.

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